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Methylphenidate effects after one-week exposure in adolescent rats

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Publications and communications

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Abstract

Methylphenidate (MPH) is a first-line stimulant drug available worldwide to treat attention deficit hyperactivity disorder (ADHD). ADHD is one of the most prevalent neuropsychiatry disorders in children and adolescents. Despite the already proven therapeutic efficiency, concerns have been raised regarding the possible consequences of MPH exposure during childhood and adolescence. Previous animal studies have focused on MPH's possible neurotoxicity but many do not correlate to the clinical use of MPH since high doses are administrated via intraperitoneal or intravenous route. Moreover, MPH effects on the peripheral organs have been scarcely studied.

This dissertation aimed to evaluate the MPH effects to the brain and peripheral organs of adolescent rats using an oral dose scheme that mimics the therapeutic doses administered to human adolescents.

Adolescent male Wistar rats (postnatal day 40) received two doses of MPH (5 mg/kg in a 5% sucrose solution), orally, 5 hours apart, for seven days, while controls received equal volume of 5% sucrose using the same scheme. Body temperature, weight and food/water intake were measured daily. Twenty-four hours after the last administration, rats were sacrificed and brain areas [cerebellum, prefrontal cortex (PFC), hippocampus, and striatum], peripheral organs (liver, heart, and kidneys), and blood were collected for posterior analysis. The body temperature, weight gain and food/water consumption were similar between controls and MPH-treated animals. In the brain, MPH significantly increased the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio in the PFC and hippocampus. However, quinoprotein levels were significantly increased in the cerebellum of MPH-treated rats. No significant changes were found in adenosine triphosphate (ATP) or protein carbonylation levels after the MPH treatment in any evaluated brain area. In the peripheral organs, no differences were found regarding ATP, quinoprotein, or protein carbonylation levels. However, the GSH/GSSG ratio was significantly increased in the heart of MPH-treated rats. The histological examination of MPH-treated rat hearts revealed significant damage, namely interstitial edema, vascular congestion, and presence of fibrin-like material. In the kidneys, necrotic areas with cellular disorganization and cell infiltration were found following MPH treatment. The lack of severe cellular damage and/or necrosis in the liver or heart of MPH-treated rats was corroborated by the absence of changes in the plasma levels of several markers (alanine aminotransferase, aspartate aminotransferase, creatine-kinase MB, and total creatine kinase). Furthermore, the amount of fibrous tissue was assessed by the Picrosirius Red staining and no differences were found among groups.

Altogether, these results suggest that one-week exposure to pharmacological relevant oral doses of MPH protected the PFC, hippocampus, and the heart from oxidative stress but, on the other hand, compromised the normal tissue organization of the heart and kidneys. Thus, further investigation is needed to discover the underlying mechanisms of these changes on adolescent animals, especially because a lifetime administration or a prolonged exposure of MPH is required in ADHD treatment.

Keywords: methylphenidate, adolescent rat, brain, peripheral organs, histology, toxicity.

Resumo

O metilfenidato (MPH) é um estimulante usado como primeira escolha terapêutica em todo o mundo para o tratamento da perturbação de hiperatividade/défice de atenção (PHDA). A PHDA é uma das perturbações neuropsiquiátricas mais prevalentes em crianças e adolescentes. Apesar da comprovada eficácia terapêutica, têm sido levantadas questões relativamente às possíveis consequências da exposição ao MPH durante a infância e a adolescência. Os estudos em animais têm-se centrado na possível neurotoxicidade deste fármaco, mas muitos investigadores usam doses elevadas administradas via intraperitoneal ou intravenosa que não se relacionam com o uso clínico do MPH. Além disso, os efeitos do MPH nos órgãos periféricos têm sido pouco estudados.

Esta dissertação teve como objetivo avaliar os efeitos do MPH no cérebro e nos órgãos periféricos em ratos adolescentes após um protocolo experimental que imita o regime terapêutico oral adotado em humanos adolescentes.

Ratos Wistar machos adolescentes (40 dias de idade) receberam duas doses de MPH (5 mg/kg dissolvidos numa solução de sacarose a 5%), via oral, com cinco horas de intervalo, durante sete dias, enquanto os controlos receberam um volume igual de sacarose a 5% no mesmo esquema. A temperatura corporal, o peso, a ingestão de água e o consumo de alimentos foram medidos diariamente. Vinte e quatro horas após a última administração, os ratos foram sacrificados e as áreas cerebrais [cerebelo, córtex pré-frontal (PFC), hipocampo e estriado], os órgãos periféricos (fígado, coração e rins) e o sangue foram recolhidos para posterior análise. A temperatura corporal, o peso, a ingestão de água e o consumo de alimentos foram semelhantes entre os controlos e os animais tratados com MPH. No cérebro, o MPH aumentou significativamente o rácio glutationa reduzida/glutationa oxidada (GSH/GSSG) no PFC e no hipocampo. No entanto, houve um aumento significativo dos níveis de quinoproteínas no cerebelo dos ratos tratados com MPH. Não foram encontradas diferenças no que diz respeito aos níveis de adenosina trifosfato (ATP) e de carbonilação proteica em todas as áreas cerebrais em comparação com o controlo. Nos órgãos periféricos, não foram observadas diferenças nos níveis de ATP, quinoproteínas e carbonilação proteica entre grupos. No entanto, o rácio GSH/GSSG aumentou significativamente no coração dos ratos tratados com MPH. A observação histológica do coração revelou danos significativos, nomeadamente edema intersticial, congestão vascular e a presença de um material semelhante à fibrina. Nos rins, foram encontradas áreas necróticas, desorganização celular e infiltração celular após o tratamento com MPH. A ausência de dano celular grave e/ou necrose no fígado e

no coração dos ratos tratados com MPH foi corroborada pela ausência de diferenças nos níveis plasmáticos de vários marcadores (alanina aminotransferase, aspartato aminotransferase, creatina quinase MB e creatina quinase total). Além disso, a quantidade de tecido fibroso foi avaliada pela coloração “Picrosirius Red” e não foram encontradas diferenças nos órgãos entre os animais controlos e tratados.

Analisando todos os resultados, estes parecem sugerir que um tratamento de uma semana com doses orais de MPH farmacologicamente relevantes protegem o PFC, o hipocampo e o coração contra o stress oxidativo mas, por outro lado, compromete a organização morfológica do coração e rins. Assim, mais estudos em animais adolescentes são necessários para avaliar os mecanismos subjacentes às alterações encontradas especialmente porque, no tratamento da PHDA, o MPH é administrado durante longos períodos ou durante toda a vida.

Palavras-chave: metilfenidato, ratos adolescentes, cérebro, órgãos periféricos, histologia, toxicidade.

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Abbreviations

5-HT – Serotonin

5-HTT – Serotonin transporter

ADHD – Attention deficit hyperactivity disorder

ALT – Alanine aminotransferase

AMPH – Amphetamine

AST – Aspartate aminotransferase

ATP – Adenosine 5'-triphosphate

AUC – Area under the plasma concentration-time curve

BP – Blood pressure

BSA – Bovine serum albumin

C_{max} – Peak plasma concentration

CNS – Central nervous system

CK-MB – Creatine-kinase MB

DA – Dopamine

DAT – Dopamine transporters

DSM – Diagnostic and Statistical Manual of Mental Disorders

DTNB – 5,5'-dithiobis(2-nitrobenzoic acid)

EDTA – Ethylenediaminetetraacetic acid

ER – Extended release

FDA – Food and Drug Administration

fMRI – Functional magnetic resonance imaging

GSH – Reduced glutathione

GSHt – Total glutathione

GSSG – Oxidized glutathione

h – Hours

HCl – Hydrochloride

$HClO_4$ – Perchloric acid

HR – Heart rate

i.p. – Intraperitoneal administration

IR – Immediate release

i.v. – Intravenous administration

$KHCO_3$ – Potassium bicarbonate

MDMA – 3,4-Methylenedioxymethamphetamine

min – Minutes

MRI – Magnetic resonance imaging

MPH – Methylphenidate

NA – Noradrenaline

NaCl – Sodium chloride

NAT – Noradrenaline transporters

$O_2^{\bullet-}$ – Superoxide anion radical

OD – Optical density

PBS – Phosphate buffered saline solution

PET – Positron emission tomography

PFC – Prefrontal cortex

PND – Postnatal days

RA – Ritalinic acid

ROS – Reactive oxygen species

SD – Standard deviation

SDS – Sodium dodecyl sulphate

SHR – Spontaneously hypertensive rats

SNAP25 – Synaptosomal-associated protein 25

$t_{1/2}$ – Half-life

Total-CK – Total creatine kinase

VMAT-2 – Vesicular monoamine transporter 2

Part I

Introduction

1. Introduction

1.1. Attention Deficit Hyperactivity Disorder (ADHD)

Attention deficit hyperactivity disorder (ADHD) is particularly relevant nowadays since it is one of the most common neuropsychiatric disorders in school-aged children and adolescents (Matthews et al., 2014). Although this complex disorder is presently known by the impulsivity and/or inattention and hyperactivity, the clinical characterization and conceptualization of ADHD suffered alterations over the last 200 years (Lange et al., 2010, APA, 2013, Matthews et al., 2014).

The first example of a disorder that resembled ADHD was described in 1798, when Crichton defined attention, and distinguished the abnormal inattention observed in children with school learning difficulties (Lange et al., 2010). However, it was in 1902 that George Still first described ADHD in his series of three published lectures to the Royal College of Physicians (Still, 1902).

Over the years, the clinicians eventually adopted different terms like “minimal brain dysfunction” (Clements, 1966), “hyperkinetic reaction of childhood” (APA, 1968), and “attention deficit disorder with or without hyperactivity” (APA, 1980) to describe the symptoms of ADHD. In 1987, the disorder was finally renamed “attention deficit hyperactivity disorder” in the Diagnostic and Statistical Manual of Mental Disorders (DSM), third revised edition and all three key features of ADHD (impulsivity, inattention, and hyperactivity) were specified (APA, 1987).

The fourth edition of DSM published in 1994 brought the distinction between two, yet correlated, domains of behavior: a set of symptoms for inattention and a set of symptoms for hyperactive/impulse behavior that allowed the diagnosis of three subtypes of ADHD: predominantly inattentive, predominantly hyperactive/impulsive, and a combined type, according to a list of criteria established by that manual (APA, 1994).

The latest characterization of the disorder is present in the DSM fifth edition. According to this edition, ADHD is described as “*a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with development, has symptoms presenting in two or more settings (e.g. at home, school, or work), and negatively impacts directly on social, academic or occupational functioning*” (APA, 2013). The description is followed by the guidelines used to correctly diagnose a particular ADHD subtype: symptoms must have onset before the age of 12 and children must exhibit at least 6 symptoms from the inattention group (e.g. easily distracted, poor concentration, difficulty completing tasks), from the hyperactivity/impulsivity (e.g. difficulty waiting and remaining seated, talk

excessively) or both for at least 6 months (APA, 2013, Sharma and Couture, 2014). The main difference of this edition when compared with the previous editions is the acknowledgement of a persistent ADHD throughout the patient's life. ADHD prevalence in children and adolescents ranging from 4 to 17 years old is presently 5% worldwide (APA, 2013). Although symptoms may decrease with age, in some cases, they can persist and the prevalence rate in adulthood is estimated at approximately 2.5% (Polanczyk et al., 2007, Simon et al., 2009, Geissler and Lesch, 2011, APA, 2013, Matthews et al., 2014). For a reliable diagnosis, older adolescents and adults over 17 years only need to display 5 of the previously stipulated symptoms (APA, 2013, Sharma and Couture, 2014).

Comorbidities like anxiety disorders, oppositional defiant disorder, and major depressive disorder can lead to a misdiagnosis of ADHD since they have symptoms and characteristics very similar to ADHD. Thus, the ADHD diagnosis is a complex and a challenging process done not only by clinicians after repeated observations, but also by using reports obtained from parents, teachers or other caregivers (Sharma and Couture, 2014).

1.1.1. Etiology and pathophysiology

As previously stated, the etiology and pathophysiology of ADHD is complex and far from being completely understood. Advances in current technology allowed a more focused research based in neuroimaging, neurochemistry, and genetics and, so far, it is known that environmental factors may have an important role on ADHD development. However, it is believed, at this point, that genetics gives a strong contribution to this disorder (Cortese, 2012, Bruxel et al., 2014).

1.1.1.1. Neuroimaging studies

Differences in brain structure in children with ADHD *versus* children without ADHD were detected by neuroimaging techniques, like magnetic resonance imaging (MRI). A reduced global brain volume, most prominent in the splenium of the corpus callosum, the right caudate and the total and right cerebral volume (Castellanos et al., 2002, Valera et al., 2007) and a reduced global grey matter volume, most prominent in the right lentiform nucleus and extending to the caudate were some of the abnormalities observed through imaging (Nakao et al., 2011). Global thinning of the cortex has also been reported. In a study with 166 children with ADHD, Shaw and co-workers concluded that the ADHD group had a significantly smaller cortical thickness, especially in the medial and superior

prefrontal and precentral regions (Shaw et al., 2006). A work of the same team also referred a significant delayed cortical maturation in the middle prefrontal cortex (PFC), after a study conducted with 223 children and adolescents with ADHD based in peak cortical thickness (Shaw et al., 2007).

Structural brain connectivity is also affected in children and adult with ADHD. Through diffusor tensor imaging, disruptions in white matter integrity, mainly in the fronto-striatal circuitry, have been reported (Weyandt et al., 2013).

The anatomic brain changes seen in ADHD results in functional alterations. The activation of neural systems measured by functional MRI (fMRI) during two tasks involving executive control ("stop" task and "delay" task) showed a lower brain activity in the prefrontal systems in adolescents (12 to 18 years old) with ADHD versus control subjects (Rubia et al., 1999). Similarly, by measuring the activity of neural regions across time through task-related studies, fMRI has shown hypoactivation (i.e. reduced blood flow) in the frontoparietal and ventral attentional networks, while hyperactivation in the default networks occurred in both children and adults with ADHD (Cortese, 2012, Cortese et al., 2012).

Overall, these findings point to a frontal-striatum-cerebellum disorder that encompass regions related to attention, cognition, sensorimotor functions, and emotions (Cortese, 2012, Bruxel et al., 2014).

1.1.1.2. Molecular and genetics studies

Taking into consideration the multiple symptoms of ADHD, several altered brain structures have arisen as potential culprits of ADHD. However, none of the changes found explains by itself the etiology of ADHD, thus several theories persist at this point.

Regarding neurotransmitter alterations, deficits in dopamine (DA) and noradrenaline (NA) were observed by positron emission tomography (PET) studies and they seem involved in the pathophysiology of ADHD. Both catecholamines are involved in the control of multiple neural systems, like locomotion, cognition, working memory, arousal, and vigilance (Prince, 2008). Additionally, it is well established that DA and NA play a critical role in the PFC, a brain region with structural and functional lesions already reported in subjects with ADHD (Rubia et al., 1999, Shaw et al., 2007). The PFC network activity is fragile and extremely sensitive to small changes in catecholamine concentrations, thus, either insufficient or excessive DA and/or NA can affect the executive functions of PFC and influence some behaviors like poor attention, impulsivity and hyperactivity (Arnsten et al., 2009, Arnsten and Pliszka, 2011). It is hypothesized that

when too little DA/NA is released from presynaptic neurons, there is an insufficient activation of postsynaptic receptors D_1/α_{2A}, respectively, which can lead to distraction and impulsivity. On the other hand, when excessive DA/NA is released, there is overstimulation of the α_1, β_1, D_1 and possible D_4 receptors in postsynaptic neurons and the individual shows inattention (Sharma and Couture, 2014).

Besides neurotransmitter alterations, molecular genetic studies also imply several genes related with the catecholaminergic system in the pathophysiology of ADHD (Cortese, 2012).

The gene that encodes dopamine transporters (DAT) protein 1 (DAT1) has been largely focused on ADHD related investigation. DAT are mainly distributed in the striatum and nucleus accumbens and they are responsible for regulating dopaminergic neurotransmission. Additionally, DAT are also targets for methylphenidate (MPH) and amphetamine (AMPH), two drugs used to treat ADHD. DAT inhibition leads to an increase of the neuronal signal and synaptic DA levels and the attenuation of the symptoms (Prince, 2008, Faraone et al., 2014). Therefore, DAT1 has been a central role in ADHD etiology and pathophysiology.

Two DA receptor genes seem also strongly related with ADHD pathophysiology. DA receptor gene 4 belongs to the D_2 class of the DA receptors and it is highly expressed on frontal lobe regions, which are associated with attention and inhibition behavior. DA receptor gene 5 belongs to the D_1 class of DA receptors and it is implicated in hippocampal memory formation (Gizer et al., 2009, Faraone et al., 2014).

Another candidate gene that might be associated with ADHD is a functional polymorphism of the serotonin transporter (5-HTT) gene, which has a long and a short variant related with a faster or slower serotonin (5-HT) reuptake and a subsequent decrease or increase in the levels of active 5-HT, respectively. The genetic polymorphism linked to this transporter might play an important role since the 5-HTT gene is expressed in neural systems related to attention, memory, and motor activities (Gizer et al., 2009). Also related to 5-HT is the 5-HT_{1B} receptor. An increased aggression and impulsive behavior in knockout mice for this receptor (Brunner and Hen, 1997) caught the attention to this gene, and a significant association to the pathophysiology of ADHD has been reported (Gizer et al., 2009).

Other authors started to consider new genes as potential disruption structures in ADHD, namely the synaptosomal-associated protein 25 (SNAP25). SNAP25 is a plasma membrane protein essential for synaptic vesicle fusion and neurotransmitter release and it is also involved in synaptic plasticity and axonal growth. A hyperactive phenotype similar to ADHD is present in the *coloboma* mouse strain lacking one copy of the SNAP25 gene (Brophy et al., 2002, Gizer et al., 2009).

Altered proteins, like the ones that resulted from genes like DA receptor gene 3, dopamine beta hydroxylase, catechol-*O*-methyltransferase, α_{2A} adrenergic receptors and brain derived neurotrophic factor also seem related with ADHD but, so far, the results do not clearly point their specific role on the disorder (Gizer et al., 2009, Bruxel et al., 2014).

The role of genes in the etiology of ADHD has been also corroborated in humans, mainly family studies. Family studies showed a 2 – 8 fold increase risk of ADHD in children from parents that presented the disorder. A higher rate of symptoms in biological relatives when compared with adopted ones was also observed in adoption family studies. However, studies with twins show the most compelling data to prove the heritability of the disorder (Faraone et al., 2005, Mick and Faraone, 2008). Faraone and co-workers estimated a mean heritability equal to 76% after a review of 20 twin studies from Australia, the European Union, Scandinavia, and the United States, giving to ADHD the title of the most heritable psychiatric disorder (Faraone et al., 2005). Therefore, most studies that search for the putative changed targets responsible for ADHD are genetic studies.

Even so, ADHD heritability is lower than 100%, therefore environment factors must bear some influence in the etiology of ADHD. Prematurity/low birth weight, prenatal smoking, exposure to cocaine, alcohol or lead, and diet (i.e. nutritional deficiencies) are some of the environmental factors considered at this point. The two environmental factors that were shown a substantial body of evidence are prematurity/low birth weight and prenatal smoking with a, respectively, 2.64 and 2.39 fold increased risk for developing ADHD (Cortese, 2012, Tarver et al., 2014).

Although the majority of the research is focused on molecular genetic studies with ADHD, methodological and technical limitations make it extremely difficult to identify the exact genes, so, both gene-gene interactions and genome-wide association studies have emerged in pharmacogenetics trying to establish concrete associations between genes and the ADHD disorder (Cortese, 2012, Bruxel et al., 2014). In fact, some of the environmental factors can trigger genes susceptible to ADHD that, otherwise, would be silent. Therefore, gene-environment interactions can result in the increased likelihood to manifest this disorder. So far, some studies have already found interactions between dopaminergic genes and prenatal smoking and/or alcohol intake but further investigation is needed to replicate results and identify potential mechanisms (Nigg et al., 2010, Cortese, 2012).

1.1.2. Present pharmacological treatment of ADHD

Pharmacological treatment of ADHD is an important piece to understand the pathophysiology of the disorder. In fact, medication is usually used to interact directly with the catecholaminergic system, more specifically with DA and NA pathways. As mentioned above, both catecholamines interact with the PFC, which has massive connections to the caudate, cerebellum, motor, and sensory cortices and regulates attention, behavior, and emotions (Arnsten and Li, 2005, Arnsten and Pliszka, 2011). Since impairment of catecholamine levels in PFC influence behaviors like poor attention, impulsivity, and hyperactivity, drug therapy aims to obtain the optimal level of DA and NA for the PFC proper function and, by that, eliminate those typical symptoms of ADHD (Arnsten et al., 2009).

According to the Food and Drug Administration (FDA), there are both stimulant and non-stimulant options for pharmacological treatment of ADHD (Swanson and Volkow, 2002). Stimulants are considered first-line agents and include MPH and AMPH, which are considered equally effective with a wide margin of safety for long-term treatment (Sharma and Couture, 2014). In general, they act at the DAT and the NA transporters (NAT) given the similar chemical structure to DA and NA, as both drugs and catecholamines present the phenylethylamine moiety (Figure 1). However, neurochemical effects and mechanisms of action are distinct (Arnsten and Pliszka, 2011). Briefly, AMPH binds to DAT, NAT, and 5-HTT and leads to the release of the monoamines from presynaptic terminals, which produces a massive increase in the neurotransmitters at the synaptic cleft (Teixeira-Gomes et al., 2015). Additionally, AMPH can be transported into intraneuronal storage vesicles and deplete the monoamine content of the vesicles, since it has affinity for vesicular monoamine transporter 2 (VMAT-2) (Carvalho et al., 2012, Teixeira-Gomes et al., 2015). Lastly, AMPH is also a reversible inhibitor of monoamine oxidase, so, once inside the presynaptic terminals, it can decrease the levels of monoamines metabolites like the DA metabolite (dihydroxiphenylacetic acid – DOPAC) and augment the synaptic levels of the monoamine neurotransmitter (Teixeira-Gomes et al., 2015). Although AMPH acts at DAT, NAT, and 5-HTT, the main mechanism of action is on DAT. That action on DAT ultimately results on a major release of DA from the presynaptic nerve terminals (Teixeira-Gomes et al., 2015). Meanwhile, MPH acts as a pure inhibitor of DAT, not as a substrate like AMPH, but this will be further detailed in section 1.2..

Although stimulants are proven safe and efficient, they are not adequate for nearly 30% of ADHD patients (Spencer et al., 1996). Several reasons like nonresponsiveness or partial responsiveness to stimulants, intolerance to their side effects (i.e., insomnia,

anorexia, irritability, nausea, headache, anxiety), presence of medical issues such as tic disorders and/or any cardiac structural abnormalities, cardiomyopathy or abnormalities of the heart rhythm, and the potential abuse or dependence are point out as reasons for the therapeutic failure for ADHD treatment (Wolraich and Doffing, 2004, Budur et al., 2005, Sharma and Couture, 2014).

MPH pharmacological and adverse effects will be more deeply aproached in a subsequent section.

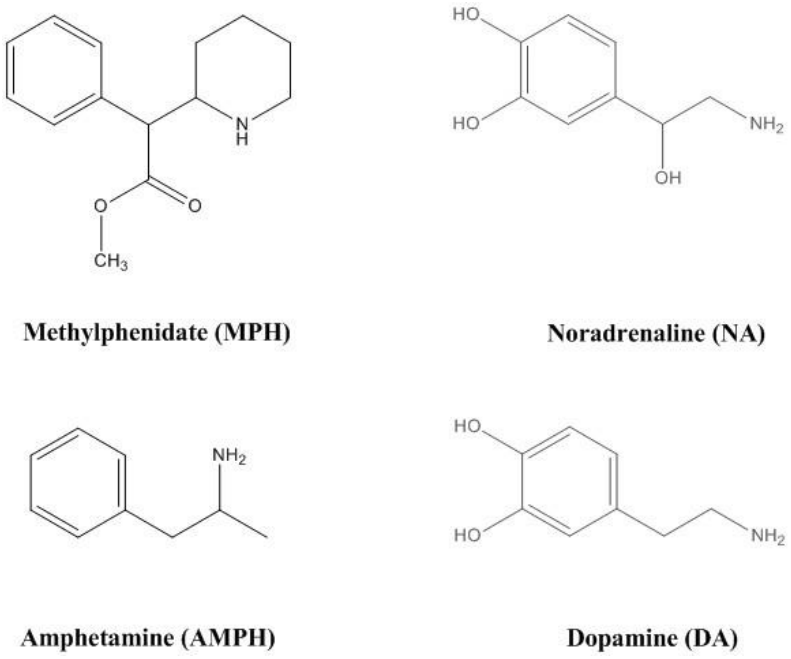

Figure 1 – Chemical structures of MPH, AMPH, and of the neurotransmitters NA and DA.

In 2002, the first non-stimulant drug, atomoxetine, was approved by FDA (Chai et al., 2012). Atomoxetine is a selective NAT inhibitor and it is capable to increase levels of both DA and NA in the PFC and improve PFC function (Bymaster et al., 2002, Sharma and Couture, 2014). Despite being considered for some authors a first-line treatment for patients with comorbid anxiety or active substance abuse disorder (Leuchter et al., 2014), FDA advises against rare cases of hepatotoxicity after its consumption (Sharma and Couture, 2014). This non-stimulant is metabolized via cytochrome P450 (CYP) 2D6 in the liver and the genetic polymorphisms in this isoenzyme can be a concern in some susceptible patients (Bolea-Alamanac et al., 2014).

Moreover, α_2 agonists can also be a therapeutic alternative for the treatment of ADHD symptoms. NA receptors are classified as α_1 (α_{1A}, α_{1B}, α_{1D}), α_2 (α_{2A}, α_{2B}, α_{2C}) and β_1, β_2 and β_3 receptors and it is known that NA has highest affinity for the α_2 receptor

family (Arnsten, 2010, Sharma and Couture, 2014). The α_{2A} subtype is the most predominant adrenergic receptor type in PFC and a disruption in this receptor or in its distribution can lead to impaired attention, poor impulse control, and hyperactivity. Therefore, it has been postulated that the use of α_2 agonists like clonidine and guanfacine can mimic NA actions on the postsynaptic receptors and control the deviant behaviors (Arnsten and Pliszka, 2011). In fact, both clonidine and guanfacine are presently used in children and adolescents aged 6–17 years in monotherapy or as adjunctive therapy when the stimulant does not fully treats the ADHD symptoms (Gormley et al., 2014).

A formulation of guanfacine extended release (ER) was approved in 2009 by the FDA (Laughren, 2009). Guanfacine, as stated, is a postsynaptic α_2 adrenergic receptor agonist that acts preferentially at α_{2A} receptors, thus, strengthening the PFC network connections. Compared to clonidine, guanfacine has 10 times less affinity for the presynaptic α_{2A} adrenoceptors, therefore, has lower rates of sedation (Dopheide and Pliszka, 2009, Bidwell et al., 2011).

Clonidine was first approved as an antihypertensive medication but, in 2010, the ER formulation of the drug was approved, by FDA, for the treatment of ADHD (FDA, 2010). It stimulates both pre- and postsynaptic α_{2A} receptors and has high affinity for all three subtypes of α_2 receptors and for imidazoline I_1 receptors, whose stimulation may mediate many of the hypotensive effects of this drug (Bidwell et al., 2011).

When both stimulant and non-stimulant drugs described above do not result in symptoms improvement, a third-line medication can be considered, that include bupropion and tricyclic antidepressants (Bolea-Alamanac et al., 2014). Bupropion is an FDA-approved drug for the treatment of depressive disorder and nicotine dependence but, it has also been used as an off-label drug to treat ADHD because it is a DA and NA reuptake inhibitor (Maneeton et al., 2014). This drug shows some advantages over MPH and AMPH, because it has a lower risk for abuse potential. Moreover, bupropion has a faster onset of action when compared with atomoxetine or α_2 agonists (Maneeton et al., 2014, Sharma and Couture, 2014). However, bupropion has a worst side effect profile and is associated with dose-related risk of seizures (Sharma and Couture, 2014). Tricyclic antidepressants have a well-demonstrated efficacy in treating impulsive and hyperactive behaviors thanks to their noradrenergic properties, but they are not so effective like stimulant drugs. Although they have some advantages over stimulants, like no risk of abuse potential and the ability to overcome depression and tics, they are third line medications because of their large number of side effects, namely sedation and life threatening overdose (Popper, 1997, Sharma and Couture, 2014).

A considerable inter-individual variability among patients implies a gradual dosage titration and a trial-and-error approach to allow the monitoring of the response to

medication, dosing, and adverse effects (Bruxel et al., 2014). Moreover, pharmacological treatments should be combined with non-pharmacological treatments as part of a comprehensive treatment plan that may include behavioral therapy, psychoeducation, lifestyle changes and diet. These approaches should include not only the patients but also their families and teachers (Tarver et al., 2014).

In the next sections, the stimulant MPH used for ADHD treatment will be addressed, since this will be the drug studied in this thesis.

1.2. Methylphenidate (MPH)

This drug was first synthesized in 1944 and it was initially indicated for chronic fatigue, depressive states, psychosis associated with depression, among others. Nowadays, it is considered the primary stimulant used to treat ADHD symptoms for its nanomolar affinity for DAT and NAT (Leonard et al., 2004, Madras et al., 2005).

MPH is a piperidine-derived molecule that contains two chiral centers and exists as four isomers, i. e., *d-threo, l-threo, d-erythro and l-erythro*-MPH. Originally, MPH was marketed as a mixture of all four isomers (80: 20 racemic *erythro* and *threo*-MPH) but subsequent investigation of the separated racemates soon eliminated the *eryhtro* of the pair, since it did not have any major activity in the central nervous system (CNS) (Challman and Lipsky, 2000, Leonard et al., 2004). Nowadays, the drug is marketed as the racemic mixture of the *threo* pair (50:50) (Heal and Pierce, 2006). Additionally, studies have shown that the *d*-isomer is the major responsible for the pharmacological activity once it is approximately 10 fold more potent than the *l*-isomer in the inhibition of DAT and NAT. Therefore, pure formulations of *d-threo*-MPH (dexmethylphenidate) have also been used since it has similar properties of the racemic mixture, but it is twice more potent (Challman and Lipsky, 2000, Wolraich and Doffing, 2004, Heal and Pierce, 2006).

Regarding the prescription of this drug, the usual daily oral dose of an immediate release (IR) formulation ranges from 10 to 60 mg (Barceloux, 2012). Along with IR formulations, which imply repeated administrations during the day, intermediate- and long-acting formulations are also prescribed. The routes of administration of these preparations are essentially two: oral or transdermal (patch in the skin) (Kaplan and Newcorn, 2011, Sugrue et al., 2014). The main features of each MPH preparation are presented in Table 1.

In the brain, more specifically in the striatum where DAT density is higher, MPH binds to the neuronal DAT and inhibits the reuptake of DA from the synaptic cleft (Volz, 2008). Usually, DAT allows the decrease in the synaptic cleft of the DA released by the

pre-synaptic neuron so, MPH by inhibiting this transporter, increases DA concentrations in the synaptic and extracellular space leading to a prolonged and/or intensified DA postsynaptic signal (Madras et al., 2005, Volz, 2008).

MPH also affects the vesicular monoamine transporter 2 (VMAT-2). In the CNS, VMAT-2 controls biogenic amines storage and it uptakes cytoplasmic DA into the synaptic vesicles to avoid oxidative deamination of the monoamine in the cytoplasm. A single administration of MPH redistributes VMAT-2 within nerve terminals from the synaptosomal membranes to the cytoplasm, which promotes sequestration of DA into the vesicles, therefore increasing the DA content available for vesicular release (Sandoval et al., 2002, Zheng et al., 2006).

Although MPH blocks more than 60% of DAT activity at oral therapeutic doses (0.3-0.6 mg/kg), it exhibits a large interindividual variability in the magnitude of increase of extracellular DA. This could be explained both by the blockade of DAT and the rate of DA release, which is regulated by individual differences in DA cell firing and by environmental stimulation (Pucak and Grace, 1994, Volkow et al., 2005).

Most of the ADHD research focused on dopaminergic system, but it is known that ADHD involves weakened PFC function and, in this brain region, DAT density is low. Therefore, it is unlikely that DAT blockade is the only responsible for PFC changes observed after MPH use (Madras et al., 2005). Therefore, new attention was brought to the NA neurotransmitter and its transporter. Indeed, several studies showed that DA affinity for the NAT is higher when compared with its affinity for the DAT. Also, NAT density was higher than DAT density in the PFC, which explains both NA and DA increase in that brain region following MPH (Madras et al., 2005).

Non-responsive patients to MPH therapy reaches in nearly 30% of ADHD patients, which might be explained by the broad mechanisms of ADHD. Non-responsiveness could reflect interindividual differences, namely very low DA activity, differences in the sensitivity or levels of DAT, differences in sensitivity of postsynaptic DA receptors and/or differences in the noradrenergic activity (Volkow et al., 2002, Volkow et al., 2005).

Table 1 – Main features of different MPH formulations available in the United States. Some of these formulations are also available in other countries.

Formulation name	Time to peak concentration (h)	Duration of action (h)	Available doses	Reference
Immediate Release				
Ritalin ®	1 – 3	3 – 4	5, 10, 20 mg	(FDA, 2013b)
Methylin ®	1 – 2	3 – 4	2.5, 5, 10 mg	(FDA, 2009c)
Focalin ® (dexmethylphenidate)	1 – 1.5	3 – 6	2.5, 5, 10 mg	(FDA, 2013a)
Intermediate–Acting				
Ritalin SR ®	4 – 5	8	20 mg	(FDA, 2013b)
Metadate ER ®	4 – 5	8	10, 20 mg	(Sugrue et al., 2014)
Methylin ER ®	3 – 4	8	10, 20 mg	(Sugrue et al., 2014)
Long-Acting (bi-modal plasma concentration-time profile, i.e., two distinct peaks approximately 4 h apart)				
Ritalin LA ®	First peak: 1 – 3 Second peak: 4 – 5	8 – 10	10, 20, 30, 40 mg	(FDA, 2013b)
Metadate CD ®	First peak: 1.5 Second peak: 4.5	8	10, 20, 30, 40, 50, 60 mg	(FDA, 2009b)
Concerta ® (osmotic release oral system)	First peak: 1 – 2 Second peak: 6 – 8	12	18, 27, 36, 54, 72 mg	(FDA, 2009a)
Focalin XR ® (dexmethylphenidate)	First peak: 1 – 4 Second peak: 4.5 – 7	12	5, 10, 15, 20, 25, 30, 35, 40 mg	(FDA, 2011)
Transdermal System				
Daytrana ®	9	Depends on usage time	10, 15, 20, 30 mg patches	(FDA, 2015)

1.2.1. Pharmacokinetics

With a low absolute bioavailability, a high volume of distribution, a high lipid solubility, a low protein binding, and a pk_a around 8.8, MPH can easily cross cellular membranes and enter the brain (Moffat et al., 2011). The pharmacokinetic parameters are dependent of the stereoselective profile, the administration route, and the formulation of the drug. Additionally, there is considerable interindividual variability, rendering difficult the evaluation of several pharmacokinetic parameters (Faraj et al., 1974, Heal and Pierce, 2006).

1.2.1.1. Absorption

Regardless of the route of administration [intraperitoneal (i.p.), intravenous (i.v.) or oral], MPH is rapidly absorbed. In case of oral administration, the absorption occurs in the gastrointestinal tract and the values of peak plasma concentration (C_{max}) and half-life ($t_{1/2}$) are dependent of the type of the formulation. For example, after the intake of IR-MPH by humans, C_{max} occurs between 1-3 hours (h) and $t_{1/2}$ between 2.6-3 h; on the other hand, ER formulations have values of C_{max} and $t_{1/2}$ slightly higher (3-4 h and 4.1 h, respectively) since these formulations are designed to assure therapeutic plasma drug levels for longer periods (Swanson and Volkow, 2002, Wolraich and Doffing, 2004, Barceloux, 2012).

MPH is also characterized by a low absolute bioavailability due to an extensive presystemic metabolism as verified by Faraj and coworkers. They investigated the disposition of MPH in man, dog, and rodents after oral or i.v. administrations (Faraj et al., 1974). The extensive presystemic metabolism was proven by higher plasma metabolites levels than MPH during the absorption phase, the pattern of urinary excretion of the radioactive isotope ^{14}C and, ultimately, by the composition of urine (Faraj et al., 1974). The bioavailability of MPH following an oral dose (10-15 mg) ranges from 11-53% in humans (Chan et al., 1983), being the bioavailability of *d-threo* significantly higher than of *l-threo* (23% and 5%, respectively) after oral administrations of three different formulations (IR, ER swallowed whole with water or ER chewed before swallowing) each with 40 mg of MPH (Srinivas et al., 1993). In rats, the bioavailability also had a wide variation (8-44%) and it was found to be 19% after oral administration when compared with an identical i.v. dose (10 mg/kg) (Wargin et al., 1983).

Due to the saturable presystemic metabolism, a nonlinear relationship between area under the plasma concentration-time curve (AUC) and oral dose was found either in studies in rats or humans (Aoyama et al., 1990, Aoyama et al., 1993). Significant

differences in other pharmacokinetic parameters like clearance, volume of distribution in steady state, AUC, and mean residence time were also reported regarding the two enantiomers (Srinivas et al., 1993). After i.v. administration of 10 mg of MPH to 11 healthy subjetcts, *l-threo* showed a clearance 2-fold higher than *d-threo* (67.99 L/h vs. 36.61 L/h), a shorter mean residence time (2.44 h vs. 6.53 h) and a volume of distribution in steady state 1.6-fold lower when compared to *d-threo* MPH (Srinivas et al., 1993).

1.2.1.2. Distribution

The tissue distribution and accumulation of MPH is rapid and occur by passive diffusion. Patrick and coworkers reported that, one minute after an i.v. administration of MPH (1 mg/kg), it was attained the peak brain concentration in male Sprague Dawley rats (Patrick et al., 1984). The accumulation was found to occur in the following magnitude: kidney > lung > brain > heart > liver 30 min after i.p. administration of MPH (20 mg/kg) in rats due to the low protein binding and high lipid solubility of MPH (Patrick et al., 1984, Wolraich and Doffing, 2004).

This pharmacokinetic parameter is affected by the stereoselective metabolism mainly after oral administration, since plasma levels of *d-threo*-MPH were significantly higher than *l-threo*-MPH 30 minutes (Yang et al.) post-administration in humans. When using i.v. administration, the difference between enantiomers was only observed 1.5 h after the administration (Srinivas et al., 1993).

Studies based on labeled *d-* and *l-threo*-MPH with ^{11}C and analysis by PET allowed to better understand the distribution of MPH in the brain. At both 30 and 100 min after oral administrations in rats, Ding and coworkers reported a higher total fraction of radioactivity in the brain after administration of *l*-isomer labeled with ^{11}C than after *d*-isomer. Also, a higher uptake of *d-threo* was observed in the striatum, but not in the cerebellum following i.v. administration of *d*-isomer when compared with *l*-isomer (Ding et al., 2004). In addition, Faraj *et al*. detected an overall preferential uptake and accumulation of MPH to the brain when compared with plasma (Faraj et al., 1974).

In humans, Volkow and coworkers showed a higher concentration of *dl-threo*-MPH in the striatum than in cortex or cerebellum, and estimated that approximately 8% of the dose (0.5 mg/kg via i.v.) entered into the human brain, reaching the peak uptake between 4 to 10 min and the half peak clearance after 90 min (Volkow et al., 1995, Swanson and Volkow, 2002).

These results evidence the striatum is the brain region where this isomer reaches higher levels (Swanson and Volkow, 2002).

1.2.1.3. Metabolism

MPH suffers metabolic reactions like de-esterification, microsomal oxidation, and conjugation to give rise to, respectively, ritalinic acid (RA), *p*-hydroxy-MPH or oxo-MPH (also named lactam) and conjugated metabolites. In addition, both *p*-hydroxy-MPH and oxo-MPH can be transformed in *p*-hidroxy-RA and oxo-RA (Faraj et al., 1974, Kimko et al., 1999). The major metabolites are pharmacologically inactive but *p*-hydroxy-MPH is relatively lipid soluble, so, it is capable to cross the blood-brain barrier and may have some pharmacological activity (Hungund et al., 1979). The main metabolic pathways for MPH are depicted in Figure 2.

In the rat, aromatic hydroxylation seems to be the predominant metabolic pathway since approximately one half of the metabolites found in the urine were *p*-hydroxy-MPH, *p*-hidroxy-RA and a glucuronide conjugate of *p*-hidroxy-RA. However, the products of de-esterification and microsomal oxidation were also detected (Faraj et al., 1974). In humans, de-esterification is the major pathway responsible for MPH metabolism, but other pathways, like aromatic hydroxylation, microsomal oxidation, and conjugation give minor contributions (Faraj et al., 1974, Redalieu et al., 1982, Kimko et al., 1999).

De-esterification is carried out by human liver carboxylesterase CES1A1, one of the most abundant carboxylesterase present in the liver and also in the gastrointestinal system. It shows a high catalytic efficiency for MPH, however, with different affinities for each enantiomer. CES1A1 carboxylesterase has a higher catalytic affinity for *l-threo* than for *d-threo*, which is consistent with the idea of a stereoselective metabolism of MPH (Sun et al., 2004, Heal and Pierce, 2006).

The MPH metabolism is very fast since RA and MPH have peak levels at approximately the same time. With poor lipid solubility, RA is easily excreted. Secondary metabolites like *p*-hidroxy-RA and oxo-RA can also be excreted unchanged or conjugated with glucuronic acid (Faraj et al., 1974, Egger et al., 1981, Wargin et al., 1983).

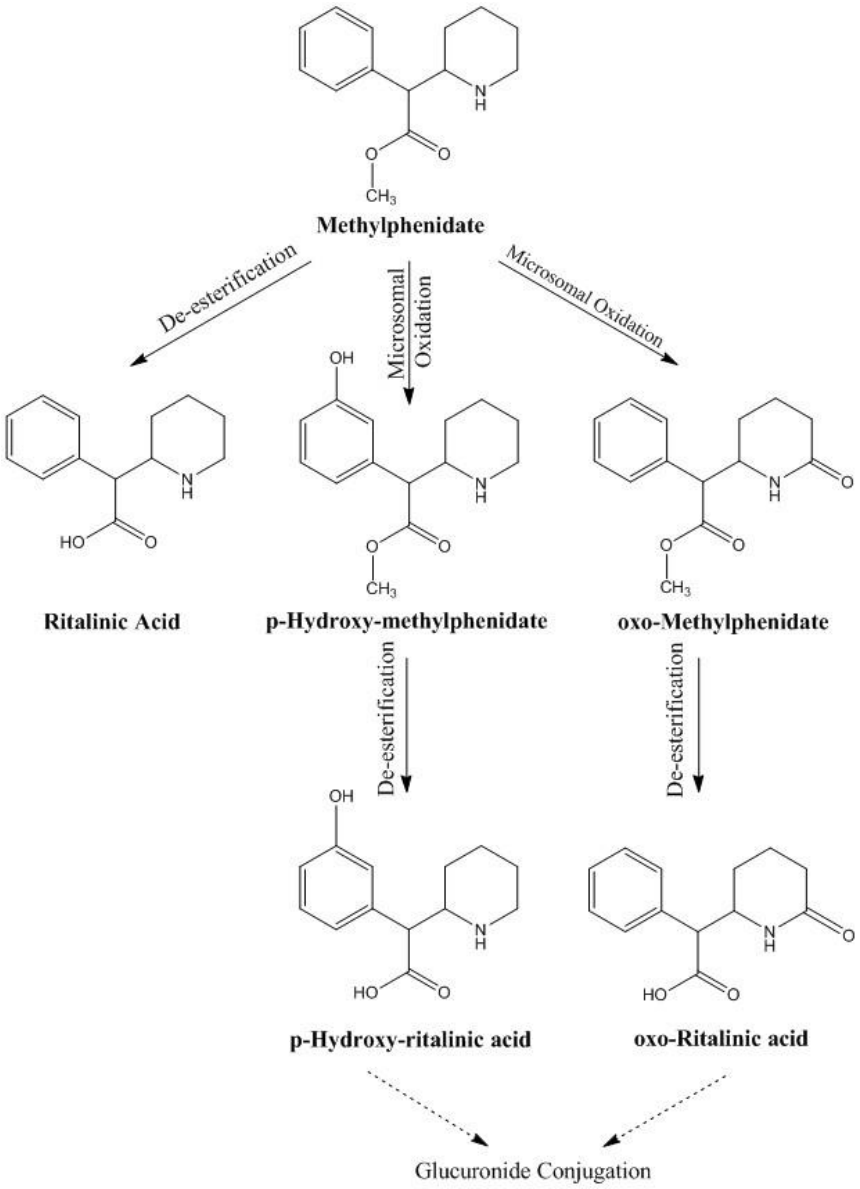

Figure 2 – Main metabolic pathways of MPH and its metabolites.

1.2.1.4. Excretion

Urine is the major route of excretion for MPH and its metabolites but two other routes (bile and feces) also contribute to MPH excretion in rats (Faraj et al., 1974, Heal and Pierce, 2006). In rats, and based on ^{14}C excretion, 48 h after i.p. or oral administration of *dl-threo*-MPH hydrochloride (HCl), 50 to 60% of marked ^{14}C was excreted in the urine and 30 to 40% in feces. After a single dose via i.p., 25 to 30% of the dose was excreted in bile within 12 h. In humans, after oral administration, 90% of the ^{14}C was excreted in the urine 48 h post-administration (Faraj et al., 1974).

About 80% of the dose found in urine of humans is RA, the main MPH metabolite (Faraj et al., 1974). Significantly higher concentrations of the *l*-RA isomer than of the *d*-RA

were found in the first 2 h following oral administration of *dl-threo*-MPH HCl (40 mg) to humans, although that difference was not observed after i.v. administration of 10 mg of the same racemic mixture (Srinivas et al., 1992).

Unchanged drug was also excreted in the urine but there are differences depending on the administration route. Between 0 and 2 h after oral administration of MPH in humans, the unchanged *d*-isomer was found in urine at 10 fold higher levels than the isomer *l*-; however, after i.v. administration, no evidence for enantioselective excretion was reported (Srinivas et al., 1992).

1.3. Acute adverse effects of MPH

Over recent years, the dramatic rise of patients diagnosed with ADHD and the increasing number of MPH prescriptions have raised concerns about the potential illicit use of this drug. In fact, MPH is classified as a Schedule II substance by the Drug Enforcement Agency, due to a high abuse potential that can lead to severe dependence (DEA, 2016). Moreover, accordingly to the Australian NSW Poisons Information Center, the number of intoxication reports with ADHD medication has increased dramatically during the recent years, being 62% of the reports associated to MPH use in the period between 2004 and 2014 (Cairns et al., 2016). Among the reasons to the misuse of this drug, the most frequent is the recreational purpose or to enhance academic performance. Since MPH has psychostimulant properties, it triggers symptoms like euphoria, greater sociability, behavioral disinhibition, and increased attention and concentration (Morton and Stockton, 2000, Cairns et al., 2016). Intentional overdoses in suicide attempts were also reported, and might be related to the high availability of MPH. Several case reports describe suicide attempts with the ingestion of a wide range of MPH doses by adolescents (210 – 1350 mg) (Fettahoglu et al., 2009, Klampfl et al., 2010, Ozdemir et al., 2010, Murat and Arman, 2013). Overdoses in preschoolers are also frequent following accidental ingestion caused by repeated or incorrect self-administered doses or given by a caregiver, and even caused by ingestion of the medication of a sibling (Foley et al., 2000). Although 93% of these intoxications results of oral ingestion of MPH, other routes like nasal insufflation of crushed tablets and i.v. administration are used to obtain the desired effects (Scharman et al., 2007, Cairns et al., 2016).

The manifestations of MPH acute toxicity results in symptoms similar to those observed after acute AMPH since these stimulants act at the same monoamine transporters (Morton and Stockton, 2000, Teixeira-Gomes et al., 2015). Neurological effects include irritability, euphoria, delusions, hallucinations, psychosis, aggressive

behaviors, and seizures. Additionally, cardiovascular effects like tachycardia, hypertension, and chest pain are also manifested (Morton and Stockton, 2000, Scharman et al., 2007). Although MPH intoxication symptoms are principally related to the CNS and cardiovascular system, other symptoms like fever, abdominal pain, and vomiting are also reported (Scharman et al., 2007). Hyperthermia, one of the most dangerous effect that can lead to rhabdomyolysis, acute renal failure, disseminated intravascular coagulation, multiple organ failure and, consequently, death have also been reported after severe intoxications of MPH (Scharman et al., 2007, Peyre and Delorme, 2012, Teixeira-Gomes et al., 2015).

1.4. Long-term adverse effects of MPH

The worldwide rise of MPH prescriptions has also created substantial concerns regarding the risks to the exposure to this stimulant since a prolonged exposure throughout the childhood or a lifetime administration is required in ADHD treatment. In 2009, the Agency's Committee for Medicinal Products for Human Use concluded that a lack of information on the long-term effects of MPH existed and that further research would be important to focus on the neurological and cardiovascular effects of patients that took or were taking MPH for the treatment of ADHD (EMEA, 2009). Actually, the CNS and the heart share several characteristics, namely low regenerative ability of some cells, excitability of some cell groups, and are both largely susceptible to differences in biogenic amines (Costa et al., 2013). These characteristics make both the CNS and the heart susceptible to some xenobiotics, like psychostimulants. Below are detailed studies regarding the adverse long-term effects of MPH in rats and humans.

1.4.1. CNS effects of MPH

Adolescence involves critical stages of neurodevelopment that can be highly influenced by psychostimulants, so, the administration of MPH can trigger long-lasting adaptive responses as a result of modifications in the internal organization and structural maturation of the brain. Moreover, these modifications can influence the behavior and the brain's capabilities during adulthood (Moll et al., 2001, Bethancourt et al., 2009). Since studies involving children or adolescents are scarce for ethical reasons, most of the data existing on the CNS effects of MPH lies in studies with laboratory animals, namely rats between postnatal days (PND) 28 and 60, period that corresponds to adolescence in this animal model (Spear, 2000).

1.4.1.1. CNS effects of MPH on laboratory animals

It is well established that repeated exposure to stimulants, like cocaine (which, like MPH, is a DAT inhibitor), is associated to significant neuroadaptations in the dopaminergic system. In fact, several authors evaluated neurophysiological changes after MPH chronic exposure and reported declines in brain DAT density (Moll et al., 2001, Marco et al., 2011). Moll and coworkers used ligand-binding-assays to study DAT density in the striatum of Wistar rats 6 or 31 days after administration of MPH (2 mg/kg/day), via the drinking water, during 2 weeks to very young (PND 25) or late adolescent (PND 50) rats. It was observed that striatal DAT density declined by 25% 6 days after the discontinuation (PND 45), and about 50% after 31 days (PND 70) in the very young group of rats (MPH administrated at PND 25). These declines were not observed in the older rats. NAT and 5-HTT were also studied but no differences were found between control and MPH treated-rats (Moll et al., 2001).

Differences in the striatal volume and myelination of adolescent and adult rats were also reported in the developing dopaminergic system after MPH administration. MPH (5 mg/kg, HCl salt) was orally administrated once daily for 21 days to Wistar rats with PND 25 or PND 65 ± 4 days, and the brains were evaluated 1 week post-administrations (van der Marel et al., 2014). The results revealed a reduction of 1.6% in striatal volume, a decrease of 9.7% in myelin content in PND 25 animals, a 2.8% increase of striatal volume, and a 3.1% increase of myelin content in adult animals when compared to the respective controls (van der Marel et al., 2014).

MPH also affects the structure and function of the brain, especially in hippocampus, which is involved in learning and memory processes (Banihabib et al., 2014). In adult rats treated with MPH (3 or 10 mg/kg, orally), once daily, for 6 days, hippocampal necrosis was detected after the higher dose (Banihabib et al., 2014). Regarding adolescent rats, a significant loss of neurons and astrocytes in the hippocampus was reported by Schmitz and coworkers (Schmitz et al., 2016). MPH (2.0 mg/kg) was administrated via i.p., once daily, between PND 15 and PND 45 and, 24h after the last administration, a significant decrease in the number of neurons and astrocytes was detected by flow cytometry when compared to the control group. Further studies were made in order to understand the underlying mechanisms of that cell death; however they only concluded that it did not involve necrosis (Schmitz et al., 2016).

The increase of extracellular DA caused by MPH may also induce oxidative stress, since DA and NA can form reactive quinones or react with molecular oxygen and Fe^{2+} to

form reactive species, namely hydroxyl radical, superoxide anion radical ($O_2^{\bullet-}$) or H_2O_2 (Gomes et al., 2009, Schmitz et al., 2012). After i.p. administration of 1.0, 2.0 or 10 mg/kg of MPH (HCl salt) once daily to young (PND 25) or adult (PND 60) Wistar rats during 28 days it was observed, 2 h after the last injection, a dose-dependent increase on lipid peroxidation and protein carbonyl formation in the cerebellum, PFC, hippocampus, and striatum of young rats but not in adult rats (Martins et al., 2006). The authors performed another experiment using the same doses but evaluated the effects acutely: 1.0, 2.0 or 10 mg/kg of MPH (HCl salt), i.p., was given only once to young (PND 25) or adult (PND 60) rats and the sacrifice was performed 2 h later. Among the adult groups, no differences were found, but in the young rats, lipid peroxidation decreased in the PFC, cerebellum, hippocampus, and striatum when compared to the respective control group (Martins et al., 2006). Oxidative stress in the hippocampus after chronic treatment with high doses of MPH was reported by Motaghinejad and coworkers (Motaghinejad et al., 2016). Male Wistar rats with 8 weeks old received 2.0, 5.0, 10, and 20 mg/kg of MPH, via i.p., for 21 days and 24 h after the treatment, lipid peroxidation and oxidized glutathione (GSSG) content increased in a dose-dependent manner, while reduced glutathione (GSH) content decreased (Motaghinejad et al., 2016). On the other hand, Schimitz and coworkers reported increased lipid peroxidation and protein damage in the PFC, but not in the cerebellum, striatum or hippocampus after Wistar juvenile rats received 2.0 mg/kg of MPH via i.p. once a day from PND 15 to PND 45 (Schmitz et al., 2012). These results argue for a higher susceptibility of the younger rat brain to redox changes induced by MPH, and that these changes are dose and time of exposure dependent.

The production of $O_2^{\bullet-}$ in submitochondrial fractions was also evaluated to allow a better understanding of the toxicity mechanisms elicited by MPH treatment. Different doses of MPH (1.0, 2.0 or 10 mg/kg) were administered, via i.p., only once or chronically (during 28 days) to Wistar rats with PND 25 or PND 60 and the production of $O_2^{\bullet-}$ in submitochondrial fractions of several brain areas was evaluated 2 h after the last administration (Gomes et al., 2009). The results revealed: (1) an increased production of mitochondrial $O_2^{\bullet-}$ in the cerebellum (in all doses) and in the hippocampus (10 mg/kg) after acute administration in young rats; (2) a decreased production in the cerebellum (1.0 and 2.0 mg/kg) after chronic administration in adult rats and, (3) no effects after chronic treatment in young rats or after acute treatment in adult rats. These results allowed the authors to conclude that MPH can influence the production of $O_2^{\bullet-}$ in the brain but that effect is age and treatment dependent (Gomes et al., 2009).

With the increase of reactive species, the enzymatic antioxidant defense system may be upregulated allowing the changes in antioxidant enzymes to prevent oxidative stress (Schmitz et al., 2012). Since oxidative stress was already reported after MPH

administration, authors evaluated the effects of MPH in the activity of two antioxidant enzymes, superoxide dismutase and catalase, after acute and chronic exposure (Gomes et al., 2008, Schmitz et al., 2012). MPH (1.0, 2.0 or 10 mg/kg) was administrated to young (PND 25) or adult (PND 60) Wistar rats, via i.p., only once (acute treatment) or once daily for 28 days (chronic treatment) and the animals were sacrificed 2 h after the last injection. In young rats, in the acute treatment, authors observed a decreased activity of superoxide dismutase in the PFC (1.0 mg/kg) and an increased activity in the cortex (all doses tested). A decreased activity of catalase was also observed in the hippocampus at the 1.0 mg/kg dose (Gomes et al., 2008). After chronic treatment in PND 25 rats, the activity of superoxide dismutase increased in the hippocampus and cortex (1.0 mg/kg), but the activity of this enzyme was inhibited in the striatum after the same dose (1.0 mg/kg). At the 2.0 mg/kg dose an increased superoxide dismutase activity in the cortex was observed. The activity of catalase was not altered in none of the brain areas evaluated after chronic exposure to MPH in young rats. Relatively to adult rats, MPH acute or chronic treatment did not cause any change in the activities of superoxide dismutase or catalase at any given dose (Gomes et al., 2008). In another study, MPH (2.0 mg/kg, i.p.) was administered to Wistar rats with PND 15 during 30 days and that treatment led to an increased activity of superoxide dismutase in the cerebellum and PFC, but not in the striatum or hippocampus, and an increased activity of catalase only in the cerebellum (Schmitz et al., 2012). Overall, these results suggest that the MPH dose, the administration period and the brain region are important determinants for the regulation of antioxidant enzymes (Gomes et al., 2008, Schmitz et al., 2012).

Regarding the energetic metabolism, several pathways have been discovered to be influenced by MPH administration. For example, altered brain mitochondrial activity has been reported in adult rats after acute and chronic exposure to MPH (Fagundes et al., 2010b). A single injection of MPH (1, 2 or 10 mg/kg, via i.p.) or an injection once a day for 28 days was given to adult Wistar rats (PND 60) and the results revealed that mitochondrial respiratory chain complexes I, II, III, and IV were inhibited in the hippocampus, PFC, striatum, and cerebral cortex either after the single dose or after the chronic exposure (Fagundes et al., 2010b). The exact same treatment schedule was performed in young Wistar rats (PND 25) and the results were completely different (Fagundes et al., 2010a). After the acute treatment, the results showed a reduction in the activity of complex I in the cerebellum and PFC and, after chronic treatment, the activities of complexes II and IV were increased in the younger animals (Fagundes et al., 2010a).

In order to understand why the activity of the mitochondrial respiratory chain was altered after MPH and, consequently, how ATP production could be impaired, Reus and coworkers studied the activity of two enzymes involved in the Krebs cycle: citrate

synthase and isocitrate dehydrogenase (Reus et al., 2013). MPH HCl salt (1.0, 2.0 or 10 mg/kg, via i.p.) was administrated, only once (acute treatment) or during 28 days (chronic treatment) to young (PND 25) or adult (PND 60) Wistar rats, which were sacrificed 2 h after the last injection. After analyzing the cerebellum, striatum, PFC, hippocampus, and cortex, the authors reported that chronic MPH exposure caused decrease activity on both enzymes, in all the brain areas of either young and adult animals while, after acute treatment, the activity of both enzymes was not altered in young rats while, in adult rats, only in the cerebellum a significant reduction on the enzymes was detected (Reus et al., 2013).

Regarding the neurobehavioral component, an increasing bulk of studies has reported changes after childhood and adolescent exposures to MPH that persist in adulthood (Bolanos et al., 2003, Carlezon et al., 2003, Bethancourt et al., 2009, Scherer et al., 2010, Marco et al., 2011). Among those behavioral changes, the authors reported: (1) reduced sensitivity to reward stimuli (Bolanos et al., 2003, Carlezon et al., 2003), (2) enhanced responsivity to stressful situations (Bolanos et al., 2003), (3) depressive-like responses and reduced adaptation to a familiar environment (Carlezon et al., 2003), (4) transient effects on hippocampal-sensitive memory (Bethancourt et al., 2009), and (5) cognitive impairments in spatial reference and working memory tests (Scherer et al., 2010). The experimental designs of these studies and their main findings are summarized in Table 2.

Table 2 – Experimental designs of neurobehavioral studies and the main findings after chronic exposure of infant or adolescent rats to MPH.

Animal characteristics	Dosage regimen	Neurobehavioral task evaluated	Promoted changes	Reference
Sprague Dawley rats were exposed from PND 20 to PND 35	2.0 mg/kg, i.p., twice a day, 4 h apart	Social play behavior, sucrose preference, locomotor response to a novel environment, elevated plus maze and self-grooming behavior, social interaction in an aversive environment, sexual behavior, and forced swim test. Play behavior task was conducted 5 days after the last injection. The remaining tasks were conducted 6 weeks after the last injection.	Decreased sucrose preference, increased anxiety-like behavior, more time engaged in self-grooming, and deficit in sexual behavior at adulthood.	(Bolanos et al., 2003)
Sprague Dawley rats were exposed from PND 20 to PND 35	2.0 mg/kg, i.p., twice a day, 4 h apart	Place conditioning to cocaine, forced swim test, and locomotor activity levels. Behavior tests were conducted at PND 60.	Reduced sensitivity to cocaine reward, increased depressive-like responses, and reduced habituation to a familiar environment.	(Carlezon et al., 2003)
Wistar rats were exposed from PND 27 to PND 71	2.0 mg/kg or 5 mg/kg, orally, twice a day, 6 h apart	Open-field testing, novel object recognition testing, and contextual fear conditioning and testing. Behavior tests were conducted at PND 89.	Increased latency to reach the criterion for sample object exploration and increased contextual fear memory.	(Bethancourt et al., 2009)
Wistar rats were exposed from PND 15 to PND 45	2.0 mg/kg, i.p., once a day	Morris water navigation task, reference memory task, working memory test, and open-field task. Behavior tests were conducted at PND 46.	Cognitive impairments of spatial reference and working memory tasks.	(Scherer et al., 2010)

In addition to different neurobehavioral responses according to the animal age and dose regimen, variations among different animal strains are also reported after acute or chronic treatment with MPH (Yang et al., 2003). Yang and coworkers analyzed locomotor responses and behavior sensitization or tolerance in three different rat strains: Sprague Dawley (wild type), spontaneously hypertensive rats (SHR), and Wistar-Kyoto (strain control for SHR). The SHR rat is one of the most used animal model to investigate ADHD since exhibits similar behaviors to those of ADHD subjects (Yang et al., 2003). MPH (0.6, 2.5 or 10 mg/kg) was administrated, via i.p., only once (acute treatment) to each 8-week-old strain and, 2 h after the injection, locomotor activity was evaluated. The authors observed that the lowest dose had no significant effect in the locomotor activity, but a single injection of 2.5 mg/kg induced a more intense response in SHR rats than on the Sprague Dawley or Wistar-Kyoto rats. Regarding the highest dose, a single dose of MPH induced a more intense response in Sprague Dawley rats followed by Wistar-Kyoto, with SHR rats being the least responsive (Yang et al., 2003). The locomotor activity was also evaluated after a chronic treatment: MPH (0.6, 2.5 or 10 mg/kg, i.p.) was administrated to each strain for 6 consecutive days. After a washout period of 3 days, rats were exposed to a re-challenge with the same doses (0.6, 2.5 or 10 mg/kg) at the eleventh experimental day. Once again, the authors did not observe any differences after the re-challenge with the 0.6 mg/kg. Regarding the other doses, repeated exposure to 2.5 mg/kg induced behavioral sensitization in both Sprague Dawley and Wistar-Kyoto rats and the repeated administration of 10 mg/kg induced tolerance in the same strains. The SHR only exhibited locomotor tolerance after the re-challenge with the highest dose (Yang et al., 2003).

1.4.1.2. CNS effects of MPH on humans

Although neurotoxicity studies conducted in laboratory animals are important, studies in humans, especially in adults after chronic exposure to MPH during early-age may give critical data. However, those studies are still scare and, when available, are sometimes contradictory. In order to understand the effects of MPH therapeutic oral doses in children, adolescent or adult brains with ADHD, the structural and functional neuroimaging of unmedicated subjects with ADHD, medicated subjects with ADHD and control groups have been used (Schweren et al., 2013).

Regarding the effects on brain structure, the analysis of structural MRI allowed to conclude that chronic exposure to MPH during childhood or adolescence probably attenuates specific structural brain abnormalities, like reduction in white matter volume of frontal, striatal, cerebellar and corpus callosum regions, since those reductions were

reported in unmedicated ADHD but not in medicated ADHD or in control groups (Schweren et al., 2013, Spencer et al., 2013).

Measuring brain activity while at rest or during tasks through fMRI can also reveal the effects of MPH in domains like working memory, attention, reward processing, error processing or emotional processing (Schweren et al., 2013). After acute exposure during childhood or adolescence, MPH seems to normalize brain activity in the striatum, PFC, and anterior cingulated cortex (Schweren et al., 2013, Spencer et al., 2013). Although several studies confirmed attenuation in brain activity after oral administrations of MPH, other studies revealed opposite results. Sheridan and coworkers measured, by fMRI, the neural activity of PFC and its connectivity to the basal ganglia during a working memory test and the results showed that PFC activity increased when the subjects (adolescent girls with ADHD) were off medication and decreased when they under the effects of MPH (Sheridan et al., 2010). Other authors reported a significant reduction of functional connectivity between the nucleus accumbens and the basal ganglia, the medial PFC, and the temporal cortex after a double-blind, placebo-controlled, two-way study in healthy individuals (23 to 35 years old) after a single oral dose of MPH (40 mg) (Ramaekers et al., 2013).

Relatively to long-term effects, adult subjects with ADHD that had received MPH during childhood were evaluated through fMRI and it was suggested that a normalization of brain activity can occur: differences in brain activity during emotional and reward processing are reported in adult unmedicated subjects with ADHD, but not in subjects with ADHD medicated or control groups (Schweren et al., 2013).

PET imaging has been also used for a better understanding of the possible effects of MPH. So far, only one study reported a long-term effect in adults (age mean, 30.9 years) with ADHD after a daily dose of long acting MPH formulation (1 mg/kg) during one year (Wang et al., 2013). PET imaging measured DAT availability and the authors observed an increased DAT availability of approximately 24% in the ventral striatum when compared to healthy controls. This finding may explain the decrease in treatment efficacy and symptoms exacerbation during the weekend or holidays when the patient is medication free (Wang et al., 2013).

More serious CNS events were associated to MPH after chronic treatment. An 11-year-old boy with ADHD presented visual hallucinations after three years of daily MPH (30 mg) (Porfirio et al., 2011). Another 12-year-old boy was diagnosed with cerebral arteritis after being treated with 10 mg of MPH twice daily for seven years (Trugman, 1988). The same diagnosis was given to an 8-year-old boy after he was treated with a daily dose of 20 mg of MPH for a year and a half (Schteinschnaider et al., 2000). In the three cases, the symptoms disappeared with the discontinuation of the MPH treatment.

Altogether, the data regarding MPH treatment shows that, although MPH is overall safe and improves brain function, brain toxicity is not absent in all the population, so, studies are urgent to assess the risks.

1.4.2. Cardiovascular effects

Cardiovascular events as a result of chronic exposure are also a matter of concern because it is known that MPH is associated with increased heart rate (HR) and blood pressure (BP). Moreover, the increase in monoamine neurotransmitters is known to interfere with several cardiovascular functions (Charatan, 2006, Take et al., 2008). For example, DA increases myocardial contraction, causes coronary vasoconstriction or vasodilatation and DA receptors have an important role in the cardiovascular circulation (Cavallotti et al., 2002). Moreover, oxidation of neurotransmitters and oxidative stress can be implied on cardiotoxic events like heart failure and ischemia-reperfusion injury, and therefore after continuous administration of stimulants that are potent releasers of neurotransmitters, cardiotoxicity can arise as a long term effect (Costa et al., 2011, Carvalho et al., 2012).

1.4.2.1. Cardiovascular effects of MPH on laboratory animals

Pronounced lesions on myocardial ultrastructure and degenerative ultrastructural abnormalities in cardiac mitochondria have been documented after chronic exposure to this stimulant (Henderson and Fischer, 1995, Take et al., 2008). Swiss-Webster mice received MPH (0.5, 2.5 or 5.0 mg/kg, three times a week, i.p. or 5.0 mg/kg, once a day, orally) for periods of 4 or 14 weeks. Moreover, Sprague Dawley rats received MPH (2.0, 20 or 100 mg/kg, i.p.) during periods of 3, 6, or 9 weeks prior to sacrifice. Although little or no recognizable differences were found in the myocardium of mice injected with 0.5 mg/kg, in the remaining groups (that included mice and rats) MPH induced myocardial membrane accumulations and lamellations, both highly focal and were evident at the earliest time-points as well as 12 weeks after the treatment, which suggests persistent effects in the myocardium (Henderson and Fischer, 1995). In another report, MPH (5.0, 10 or 20 mg/kg) was administered orally to PND 25 Wistar rats for 5 days/week during three months and, at the end of the third month, the left ventricular heart tissue was removed and it was analyzed by immunohistochemistry (Take et al., 2008). A dose-dependent increase of D_2 expression in the myocytes and connective tissue was observed, which

was related with mitochondria degeneration or an increased number of mitochondria that led to myofibril structure disruption and local effusion (Take et al., 2008).

1.4.2.2. Cardiovascular effects of MPH on humans

Increased levels of NA and consequent sympathetic nervous stimulation have been initially associated to the cardiovascular events seen after MPH exposure, but it has been recognized that increases of DA in the brain and of adrenaline in plasma are also involved in the cardiotoxicity of this stimulant (Volkow et al., 2003). Moreover, treatment with MPH has been associated with minor but statistically significant mean increases in BP and HR within hours, 12 weeks or even after 12 weeks of administration in children and adult with ADHD (Stiefel and Besag, 2010). After analyzing several placebo-controlled studies, Rapport and Moffitt reported an increase HR ranging from 3 to 10 beats *per* minute and an increase systolic BP and diastolic BP ranging, respectively, from 3.3 to 8 mmHg and 1.5 to 14 mmHg as a result of short-term administrations of MPH in children (Rapport and Moffitt, 2002). Meta-analysis of published clinical trials reported in adults an increase in HR of 5.7 beats *per* minute and an increase of 2 mmHg in systolic or diastolic BP, when compared with randomized placebo subjects with a mean treatment exposure of 6 weeks (minimum of 4 weeks and maximum of 24 weeks) (Mick et al., 2013).

Very serious cardiotoxic events have also been reported in different situations. Two adult men with 35 and 37-year-old suffered cardiac arrhythmias after receiving an i.v. dose of 0.3 mg/kg MPH (Lucas et al., 1986). Another 27-year-old man with adult attention deficit disorder was diagnosed with acute myocardial infarction, probably secondary to coronary vasospasm, 24 h after he ingested 25 mg of MPH (Thompson and Thompson, 2010). An 18-year-old man suffered acute dilated cardiomyopathy one year after daily intake of MPH (54 mg) (Nymark et al., 2008). Cardiac arrest with pulseless electrical activity was also reported in an adolescent after 18 months with a daily dose of 36 mg of MPH (Daly et al., 2008). Chronic exposure to MPH was also linked to cardiac arrest following myocardial infarction in an 11-year-old boy that had two years with a daily dose of 54 mg (Munk et al., 2015).

Sudden deaths were also linked to MPH administration in the data collected between January 1992 and February 2005 in the Unites States of America by the FDA (FDA, 2006). Among those cases, fourteen were children aged from 1 to 18 years and four were adult (older than 18). Their deaths were associated with MPH usage, although six pediatric cases revealed structural cardiovascular abnormalities in the autopsies that may had increased their cardiovascular risk (FDA, 2006).

Part II

Aims of the Study

2. Aims of the Study

The increasing use and abuse of MPH has raised great concerns regarding the possible long-term effects on the neurobiology and development, mainly of adolescents since their brain is not fully mature. Additionally, deleterious cardiovascular events can be induced by catecholamine excess, whereby, heart structure and function can be altered in MPH treated patients. Therefore, a better understanding of the consequences of an early exposure to this drug used in ADHD treatment is essential. Specifically, it is necessary to understand the specific neuroadaptations and cardiovascular events underlying the chronic exposure to MPH in order to prevent and/or treat them.

The main aim of this dissertation was to assess the adverse effects of this drug in an adolescent rat model (PND 40) after a one-week exposure to a dose scheme that mimics the therapeutic doses taken by adolescents during the treatment of ADHD, mainly focusing on MPH brain and cardiac effects. The effects were evaluated in four different brain areas (cerebellum, PFC, hippocampus, and striatum) and in three peripheral organs (liver, heart, and kidneys) 24 h after the last administration. To achieve the aims, several determinations like energetic content, redox status, quinoproteins formation, and protein carbonylation were performed. Additionally, histological analysis on the three peripheral organs was done and the levels of plasma enzymes of liver or heart damage was assessed.

Part III

Materials and Methods

3. Materials and Methods

3.1. Materials

MPH (HCl salt) was purchased from Tocris Bioscience (Bristol, UK). Ethylenediaminetetraacetic acid (EDTA), perchloric acid ($HClO_4$), sodium hydroxide (NaOH), sodium carbonate (Na_2CO_3), disodium phosphate (Na_2HPO_4), cupper (II) sulphate ($CuSO_4$), potassium bicarbonate ($KHCO_3$), potassium dihydrogen phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$), and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline solution (PBS) was purchased from Biochrom (Berlin, Germany), sodium chloride (NaCl), and sodium dodecyl sulphate (SDS) from VWR (Leuven, Belgium), potassium sodium tartrate from Fluka (Buchs SG, Switzerland), methanol, and xylene from Fisher Scientific (Loughborough, UK). Harris hematoxylin was purchased from Harris Surgipath (Richmond, IL, USA), eosin 1% aqueous from Biostain (Traralgon, Australia), and Histofluid from Marienfeld (Lauda-Königshofen, Germany). EMLA® Lidocaine 25 mg/g + Prilocaine 25 mg/g was obtained from AstraZeneca (London, UK). Isoflurane (Isoflo® 100% p/p) was obtained from Abbott Animal Health (North Chicago, IL, USA). ABX Pentra reagents were purchased from HORIBA (Kyoto, Japan). DC™ Protein Assay kit and the Clarity™ Western ECL Substrate were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Dinitropenhyl-KLH rabbit IgG antibody was purchased from Invitrogen/Life Technologies (Grand Island, NY, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit antibody, slot blot apparatus and 0.45 µm Amersham Protran nitrocellulose blotting membrane were purchased from GE Healthcare Bio-Sciences (Pittsburgh, PA, USA). All the other chemicals used were purchased from Sigma-Aldrich (St. Louis, MO, USA).

3.2. Animals

Fourteen adolescent male Wistar rats at PND 40 and weighing an average 120 g were born in the animal facilities of the Institute for Biomedical Sciences Abel Salazar, University of Porto (ICBAS-UP). Animals were kept in a controlled environment (23°C, 55% humidity, 12h light/dark cycles) with food and water available *ad libitum*. All procedures were performed in order to minimize animal suffering and stress. Housing and experimental treatment were based in the guidelines defined by the European Council Directive (2010/63/EU) transposed into Portugal law (Decreto-Lei n. º 113/2013).

Additionally, the experiments were approved by the Ethical Committee of the Faculty of Pharmacy, University of Porto (process n. º 17/03/2014) and the Portuguese National Authority for Animal Health (General Directory of Veterinary Medicine) (process n. º 0421/000/000/2015).

3.3. Experimental protocol

One-week prior to MPH administration, the dorsocervical region of each animal was trichotomised and anesthetized locally with a local anaesthetic (EMLA ® Lidocaine 25 mg/g + Prilocaine 25 mg/g) that acted for about 60 min. Next, under a brief inhalatory anaesthesia with isoflurane, a subcutaneous insertion of a temperature transponder (BioMedic Data Systems Inc., Seaford, DE) was made to allow precise core body temperature measurements throughout the experimental period. In the following days, animals were maintained in groups to allow socialization and experienced a daily training to the sound of the temperature measurement device. Additionally, the animals were trained to receive oral administrations, by syringe, of a 5% sucrose solution. This was done in order to ensure an easy, complete and rapid drug intake during the treatment period. Cornflakes were given along the oral administration as a reward and to make sure that the solution was really swallowed. Twenty-four hours before the experiment and for the next seven days, the animals were individually housed with continuous access to food and water *ad libitum*.

At PND 40, the fourteen male Wistar rats were randomly assigned to the two experimental groups: control (n = 7) and MPH-treated (n = 7). The MPH-treated group received two doses (one in the morning at 9 a.m., and other in the afternoon at 2 p.m., 5 h apart) of 5 mg/kg MPH previously prepared in sterile sucrose 5% solution. Controls received an equal volume of 5% sucrose in the same scheme. To more closely simulate the clinical use of MPH, we used low doses of oral MP administered to young rats divided in two daily intakes. The doses were selected on the basis of pharmacokinetic modeling to achieve peak plasma levels near the clinical range, and were previously used by other groups (Kuczenski and Segal, 2002, van der Marel et al., 2014).

Before each morning administration, animals were weighted in order to adjust the dose to the individual weight and to assess possible changes in weight. Body temperature was also monitored and registered before each dose, 30 min after the administration and, after that, every 15 min for a total of 2.5 h. Food and water intake were also measured, daily, throughout the 7 experimental days. Twenty-four hours after the last administration, rats were sacrificed and brain areas (cerebellum, PFC, hippocampus, and striatum),

peripheral organs (liver, heart, and kidneys) and blood were collected for posterior analysis.

3.4. Blood and tissue collection

The animals were anesthetized with isoflurane, a volatile anaesthetic and, before decapitation, blood was collected from the inferior vena cava to determinate the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine-kinase MB (CK-MB) and total creatine kinase (total-CK). After sacrifice, the brain was collected, weighted and dissected into four areas (cerebellum, PFC, hippocampus and striatum) according to a rat brain atlas (Swanson, 2004). The liver, heart, and kidneys were also collected and weighted.

The brain areas dissected from the left hemisphere were placed in $HClO_4$ 5% (w/v) and homogenized in a sonicator (20 seconds, continuously) while the tubes were on ice. The resulting homogenates were then centrifuged (13,000 rpm, 10 min, 4ºC) and the supernatants were aliquoted and stored at -80ºC for posterior determination of total glutathione (GSHt), GSSG, and adenosine 5'-triphosphate (ATP). The pellet was kept at -20ºC for protein determination. The brain areas dissected from the right hemisphere were collected in RIPA buffer supplemented on the day of sacrifice with protease and phosphatase inhibitors [150 mM NaCl, 50 mM Tris, 0.1% SDS (v/v), 0.5% sodium deoxycholate (m/v), 1% Triton X-100 (v/v), 0.25 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM sodium metavanadate ($NaVO_3$), 10 mM sodium fluoride (NaF), protease inhibitor cocktail from Sigma, pH = 8.0]. The samples were then homogenized in a sonicator (20 seconds, continuously) while kept on ice, and the homogenates were centrifuged (13,000 rpm, 10 min, 4ºC). The supernatants were aliquoted and stored at -80ºC for posterior analysis of quinoprotein levels and protein carbonylation.

Regarding the peripheral organs, after being weighed, a 2 mm section of each organ from four controls and four MPH-treated rats was collected and fixed in a 4% paraformaldehyde solution in PBS for further histologic analysis. A section of liver, heart, or kidneys from all animals was collected in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.25 mM PMSF, 1 mM $NaVO_3$, 10 mM NaF, protease inhibitor cocktail from Sigma, pH = 8.0). These samples were homogenized in a sonicator (30 seconds, continuously) while the tubes were on ice and the homogenates were centrifuged (5,000 rpm, 10 min, 4ºC). The supernatants were then aliquoted and stored at -80ºC for quinoprotein levels and protein carbonylation determinations. The remaining organs were homogenized in an Ultra-Turrax while in 0.1M

KH_2PO_4 (pH = 7.4). A homogenate aliquot was stored at -20ºC for protein quantification. Another aliquot was added to $HClO_4$ 10% (w/v) in a reason 1:1 and the solution was centrifuged (13,000 rpm, 10 min, 4ºC). The supernatants were then separated and stored at -20ºC for GSHt and GSSG determination and at -80ºC for ATP determination. All the procedures were performed on ice.

3.5. Measurement of plasma biomarkers

The blood from the inferior vena cava was collected into EDTA-containing tubes and, after centrifugation (920*g*, 10 min), the obtained plasma was stored at -20ºC until determination of ALT, AST, CK-MB and total-CK. The plasma biomarkers were determined through enzymatic assays in the apparatus ABX Pentra 400 with ABX Pentra reagents, according to the manufacturer's instructions. The determinations were performed by Dr[a] Laura Pereira, at the clinical analysis laboratory of the Faculty of Pharmacy of the University of Porto.

3.6. Measurement of ATP levels

ATP levels of the four brain areas and of the three peripheral organs were determined by a bioluminescent assay based in the luciferin-luciferase reaction (Capela et al., 2007, Costa et al., 2007). Aliquots of D-luciferin 90.9 mg/l stock reagent and luciferase from *Photinus pyralis* (firefly) (3 x 10^6 light units/ml) were first prepared in a luciferin-luciferase buffer [50 mM glycine, 10 mM $MgSO_4$, 1 mM Tris, 0.55 mM EDTA, 0.1% bovine serum albumin (BSA) (m/v), pH = 7.6] and stored at -80ºC, protected from light until use. These stock solutions were mixed in the day of the ATP determination. An ATP calibration curve was prepared in $HClO_4$ 5% (w/v) with concentrations ranging from 0.5 to 10 µM. At the day of the assay, 150 µl of samples, standards or blanks were neutralized with an equal volume of 0.76 M $KHCO_3$, and, after a quick centrifugation (13,000 rpm, 1 min, 4ºC), 100 µl of the supernatants were placed in a 96-well white microplate. Then, 100 µl of luciferin-luciferase reagent solution previously prepared was added to each well and bioluminescence was immediately read in a microplate reader Biotech Synergy HT (VT, USA). In order to avoid loss of bioluminescence signal and residual bioluminescence, a maximum of 6 measurements were made in each reading. The results were expressed as nmol of ATP *per* mg of protein (nmol ATP/mg protein).

3.7. Measurement of GSHt, GSH, and GSSG

The GSHt and GSSG levels of the different brain areas and the peripheral organs were evaluated by the 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB)-GSSG reductase recycling assay (Capela et al., 2007, Costa et al., 2007). For the GSHt assay, 200 µl of samples, standards or blanks were neutralized with 200 µl of 0.76 M $KHCO_3$, vortexed, and centrifuged (13,000 rpm, 5 min, 4ºC). In a 96-well microplate, 100 µl of neutralized blanks, standards or samples were added in triplicate and mixed with 65 µl of the reagent solution containing 0.68 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 3.96 mM DTNB freshly prepared in a phosphate buffer (71.5 mM Na_2HPO_4, 71.5 mM NaH_2PO_4, 0.63 mM EDTA, pH = 7.5). The 96-well microplate was then incubated in a Biotek PowerWaveX plate reader (VT, USA) for 15 min at 30ºC. After that, 40 µl of fresh glutathione reductase solution (10 U/ml in phosphate buffer) was added to each well. The stoichiometric formation of 5-thio-2-nitrobenzoic acid was monitored for 3 min at 10 seconds intervals, at 415 nm, and compared with the calibration curve. Standards were prepared in $HClO_4$ 5% (w/v) with concentrations ranging between 0.5 and 15 µM.

For the GSSG assay, before neutralization, 10 µl of 2-vinylpyridine was added to the acidic homogenates, standards or blanks and mixed for 1 h, on ice and under agitation in order to block GSH. After this period, the determination of GSSG was performed as described above for GSHt. GSSG standards were also prepared in $HClO_4$ 5% (w/v) and concentrations ranged from 0.25 to 8 µM. Moreover, the levels of GSH were calculated by the formula: GSH = GSHt – 2 x GSSG. The results of GSHt, GSH, and GSSG were normalized to the total protein content and were expressed as nmol of GSH or GSSG *per* mg of protein (nmol GSH/mg protein, or, nmol GSSG/mg protein).

3.8. Quinoprotein assay

The levels of protein-bound quinones (quinoproteins) in the brain and peripheral organs were determined by the nitrotetrazolium blue chloride (NBT)/glycinate colorimetric assay, as previously described (Capela et al., 2007). Twenty-five µg of protein lysates in RIPA buffer were added to 2 M potassium glycinate solution (pH = 10) to obtain a final volume of 250 µl. Then, 500 µl of NBT reagent (0.24 mM NBT in 2 M potassium glycinate, pH = 10) was added to start the reaction. The reaction occurred for 3 h, under agitation, at room temperature and protected from light. One hundred µl of the samples was added in triplicate and the absorbance was read at 530 nm in a 96-well microplate reader (Biotek

PowerWaveX plate reader, VT, USA) to assess the formation of the blue-purple colored complex between quinoproteins and the reagents. The results were expressed in optical density (OD) *per* mg of protein (OD/mg protein).

3.9. Protein carbonylation assay

As an index of protein oxidation, protein carbonyls in the brain and peripheral organs were determined by the reaction between 2,4-dinitrophenyllhydrazine (DNPH) and protein carbonyls and the subsequent detection of the dinitrophenyl hydrazine adduct. All samples were diluted in complete RIPA to a final concentration of 0.1 mg/ml. Next, 200 µl of SDS 12% (w/v) was added to 200 µl of samples containing 20 µg of protein. Samples were then incubated with 400 µl of 20 mM DNPH in 10% trifluroacetic acid (v/v) for 30 min, at room temperature and protected from the light. After incubation, samples were neutralized with 300 µl of neutralization solution [18% β-mercaptoethanol (v/v) in 2 M Tris] and centrifuged at 13,000 rpm for 2 min. The supernatants were diluted in PBS to obtain a final concentration of 1 ng protein/µl. Using a slot blot apparatus (Amersham Life Science, Richmond, USA), 100 µl of the derivatized proteins were then loaded, under vacuum, into the nitrocellulose membranes (Hybond ECL, Amersham Pharmacia Biotech) previously hydrated in 10% methanol for a few seconds and 2-3 min in distilled water. Next, the membranes were washed in TBS-T [20 mM Tris base, 300 mM NaCl, 0.05% Tween 20 (v/v), pH = 8.0], and blocked in the blocking buffer [5% milk (w/v) in TBS-T] for 1h, under agitation, at room temperature. The membranes were then incubated with primary antibody (rabbit polyclonal anti-DNP, 1:1000) overnight, at 4ºC. In the next day, membranes were washed five times (10 min each), under agitation, with TBS-T and the secondary antibody (anti-rabbit IgG-peroxidase, 1:2000) was added for 1h, under agitation, at room temperature. Both antibodies were diluted in the blocking buffer. Following three washes (10 min each) with TBS-T under agitation, the detection of immunoreactive bands was possible through the use of the Clarity™ Western ECL Substrate, used according to the manufacturer's instructions. Digital images were acquired by a Molecular Imager ® ChemiDocTM XRS+ System (Bio-Rad Laboratories, CA, USA) and analyzed by the Image LabTM Software (Bio-Rad Laboratories, CA, USA). Results of OD were expressed as percentage of control values (% of control).

3.10. Histological treatment and analysis

The 2 mm sections of liver, heart, and kidneys of four controls and four MPH-treated rats were fixed overnight in a solution of 4% paraformaldehyde (m/v) in PBS at 4ºC, dehydrated with grade ethanol solutions (70%, 80%, 95%, and 100%), cleared with xylene and, finally, embedded in paraffin in an oven at 56ºC. Paraffin sections with 5 µm thickness were then obtained in a manual rotator microtome Leica RM2125 (Wetzlar, Germany). The tissues were stained with hematoxylin and eosin as follows: 5 min xylene (2x), 5 min ethanol 100%, 5 min ethanol 95%, 5 min ethanol 80%, 5 min ethanol 75%, 5 min water, 5 min hematoxylin, 8 min running water, 5 min eosin, 5 min ethanol 95%, 5 min ethanol 100%, 5 min xylene, and mounted with DPX. All preparations were analyzed with a Carl Zeiss Imager A1 light microscope and images were recorded with a coupled AxioCam MRc 5 digital camera (Oberkochen, Germany).

3.11. Collagen detection and analysis

The paraffin sections of liver, heart, and kidneys were also stained with Picrosirius Red as follows to detect collagen deposition: 5 min xylene (2x), 5 min ethanol 100%, 5 min ethanol 95%, 5 min ethanol 80%, 5 min ethanol 75%, 5 min water, 90 min 0,1% sirius red dissolved in saturated aqueous picric acid, rinsed twice in 0.5% acetic acid, 5 min ethanol 95%, 5 min ethanol 100%, 5 min xylene, and mounted using DPX. All preparations were first evaluated in a Nikon Eclipse TS100 microscope with a coupled Nikon DS-Fi1 camera (Japan) and then analyzed with ImageJ (NIH, Bethesda, Maryland, USA). The results are expressed as percentage of collagen area (stains red) by muscle area (stains yellow) (% collagen/muscle area).

3.12. Protein quantification

In general, the protein content was determined by the Lowry method (Lowry et al., 1951). Protein standards were prepared with BSA in 0.5 M NaOH, and the standard curve ranged from 25 µg/ml to 250 µg/ml. To a 96-well microplate, 50 µl of samples, standards or blanks were added in triplicate and mixed with the freshly prepared reagent A (9.8 ml of 2% Na_2CO_3, 100 µl of 2% sodium potassium tartrate and 100 µl of 1% $CuSO_4$). After 10 min in the dark, 100 µl of extemporaneously prepared reagent B (Folin-Ciocalteu reagent and water, 1:14) was added and the microplate was kept in the dark for more 20 min, after

which the absorbance was measured at 750 nm in a 96-well microplate reader (Biotek PowerWaveX plate reader, VT, USA).

Regarding the samples containing complete RIPA buffer, namely for the quinoprotein and protein carbonylation assays, the protein content was determined by the DC™ Protein Assay kit from Bio-Rad, according to the manufacturer's instructions.

3.13. Statistical analysis

Results are expressed as mean ± standard deviation (SD). Statistical analysis of the temperature, as well as animal weight, food and water intake was carried out by the two-way analysis of variance (ANOVA) with repeated measurements test, followed by a Bonferroni *post-hoc* test. When comparisons were done between the two studied groups at the end of the time-point, statistical comparisons were performed by the t-test when data followed a normal distribution or by the Mann-Whitney Rank Sum test when data did not pass the normality test. Differences were considered significant at *p* values lower than 0.05. Outliers were identified using the ROUT's test. All analyses were performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

Part IV

Results

4. Results

4.1. MPH treatment had no effect on core temperature, body weight gain, and food/water intake in adolescent rats

MPH (5 mg/kg in a 5% sucrose solution) or 5% sucrose (m/v) were orally administrated, twice a day, 5 hours apart, for seven days to adolescent rats and the body temperature, weight, and food/water intake were daily measured throughout the experimental period. In Figure 3, the results of the daily temperature in the first day (Figure 3A and 3B) and seven days later (Figure 3C and 3D) are presented after each dose. No significant differences were found between the control and MPH-treated group. Daily temperature between day 2 and 6 were also analyzed and no differences were found among groups after each dose (data not shown).

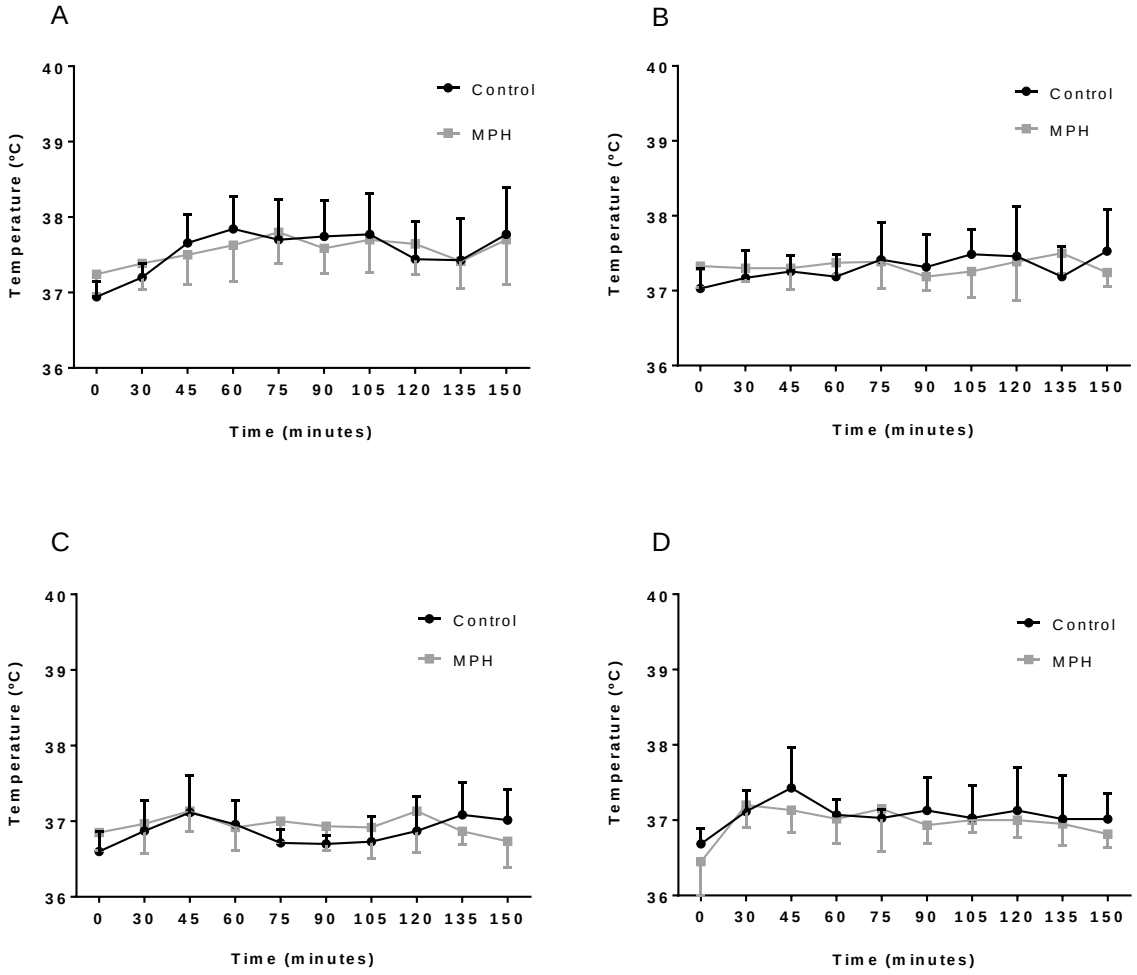

Figure 3 –Temperature monitoring of rats in the first day of the experimental protocol after the first dose in the morning (A) and the second dose in the afternoon (B), and in the last day after the first dose in the morning (C) and the second one in the afternoon (D). Results, in degrees Celsius (ºC), are expressed as mean ± SD, from seven animals in each group. Statistical analysis was made using the two-way ANOVA followed by the Bonferroni *post hoc* test.

Regarding the body weight gain *per* day and food or water intake (*per* day and *per* weight), no significant differences were found between the two studied groups. However, data showed a tendency for a decreased weight gain in the MPH-treated group (Figure 4A). Even so both food and water intake were similar between the two groups (Figure 4B and 4C, respectively).

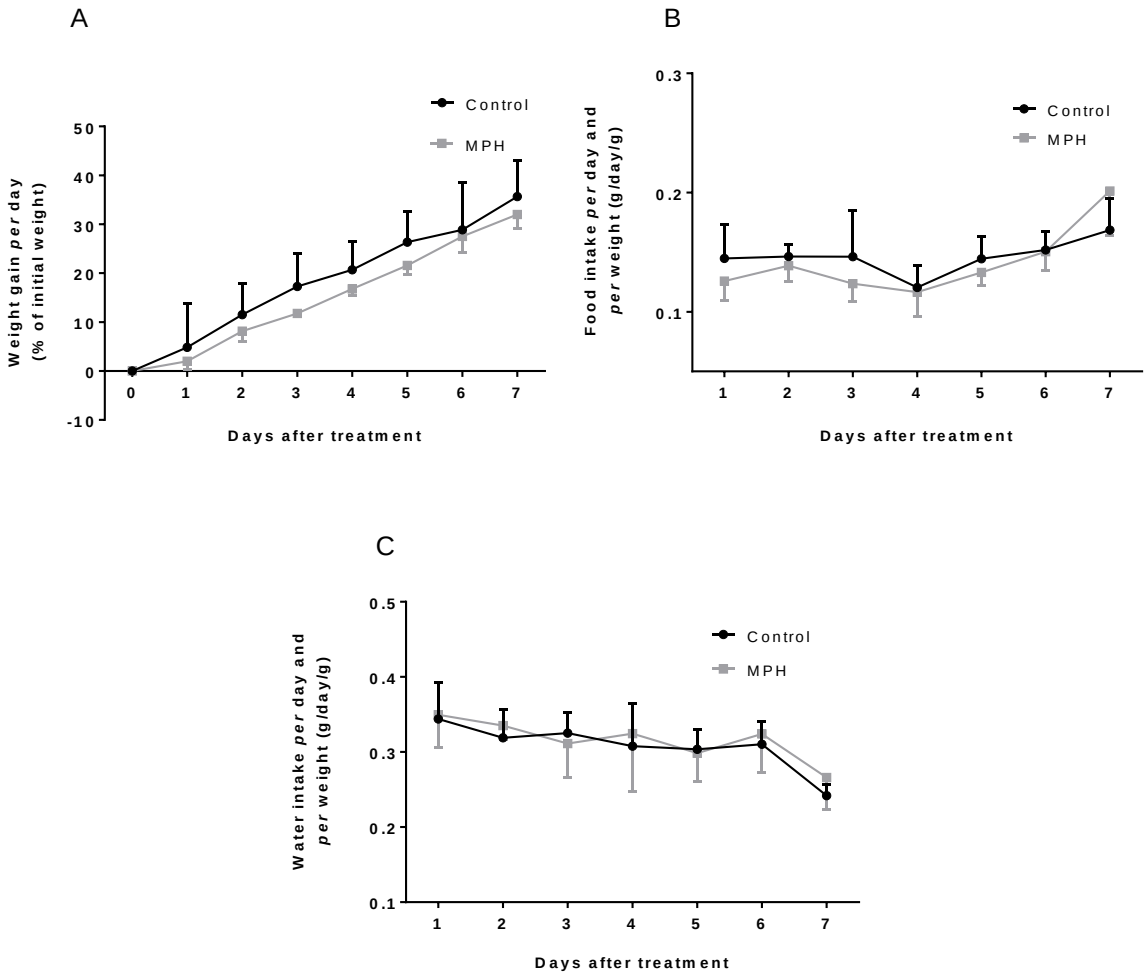

Figure 4 – Body weight gain *per* day (A), food (B), or water (C) intake *per* day and *per* weight of control and MPH-treated animals. Results in percentage of initial weight, in g/day or g/day/g, from seven animals in each group are expressed as mean ± SD. Statistical analysis was made using two-way ANOVA followed by the Bonferroni *post hoc* test.

4.2. The weight ratio of peripheral organs was not altered by MPH treatment

The weight of each collected organ (liver, heart, kidneys, and brain areas) was registered. The weight ratio of each peripheral organ was calculated in relation to the brain weight. No significant differences were observed between control and MPH-treated rats (Table 3).

Table 3 – Organ weight ratio of control and MPH-treated rats.

	Control	MPH
Liver weight/Brain weight ratio	4.47 ± 0.40	4.48 ± 0.44
Heart weight/Brain weight ratio	0.40 ± 0.04	0.42 ± 0.04
Kidneys weight/Brain weight ratio	0.88 ± 0.08	0.93 ± 0.08

Results, from seven animals in each group, are expressed as mean ± SD. The mean brain weight of the control group was 1.59 ± 0.06 g, and of MPH-treated group was 1.61 ± 0.04 g. Statistical analysis was made using the t-test.

4.3. Plasma biomarkers of liver and heart damage were not changed by MPH treatment

The levels of plasma biomarkers in control and MPH-treated rats were determined in order to detect liver (ALT and AST) or heart damage (AST, CK-MB, and total-CK). The results showed no significant differences for any biomarker measured 24 h after the exposure to MPH during seven consecutive days (Table 4).

Table 4 – Biochemical plasma biomarkers of control and MPH-treated rats.

	Plasma Levels	
Biomarker	**Control**	**MPH**
AST (U/L)	166 ± 78	122 ± 37
ALT (U/L)	39 ± 9	38 ± 5
AST/ALT ratio	4.14 ± 1.42	3.17 ± 0.72
CK-MB (U/L)	664 ± 375	809 ± 157
Total-CK (U/L)	788 ± 549	964 ± 377

Plasma levels of AST, ALT, CK-MB, and total-CK of control and MPH-treated group. Results, in units per liter (U/L), are expressed as mean ± SD. Data were obtained from seven animals in each group, except for the CK-MB and total-CK levels of the control group that were obtained from five animals. Statistical analysis was made using the t-test for AST and ALT levels, and the Mann-Whitney Rank Sum test for CK-MB and total-CK levels.

4.4. ATP levels in the PFC show a tendency to decrease after MPH treatment

The ATP levels were determined 24 h after the seven days treatment and showed no significant differences in the cerebellum, hippocampus, and striatum, as can be seen in Figure 5A, 5C, and 5D, respectively. However, data showed a strong tendency for a decrease (p = 0.051) in ATP levels in the PFC (Figure 5B) of adolescent rats after oral administration of MPH (5 mg/kg) for seven days.

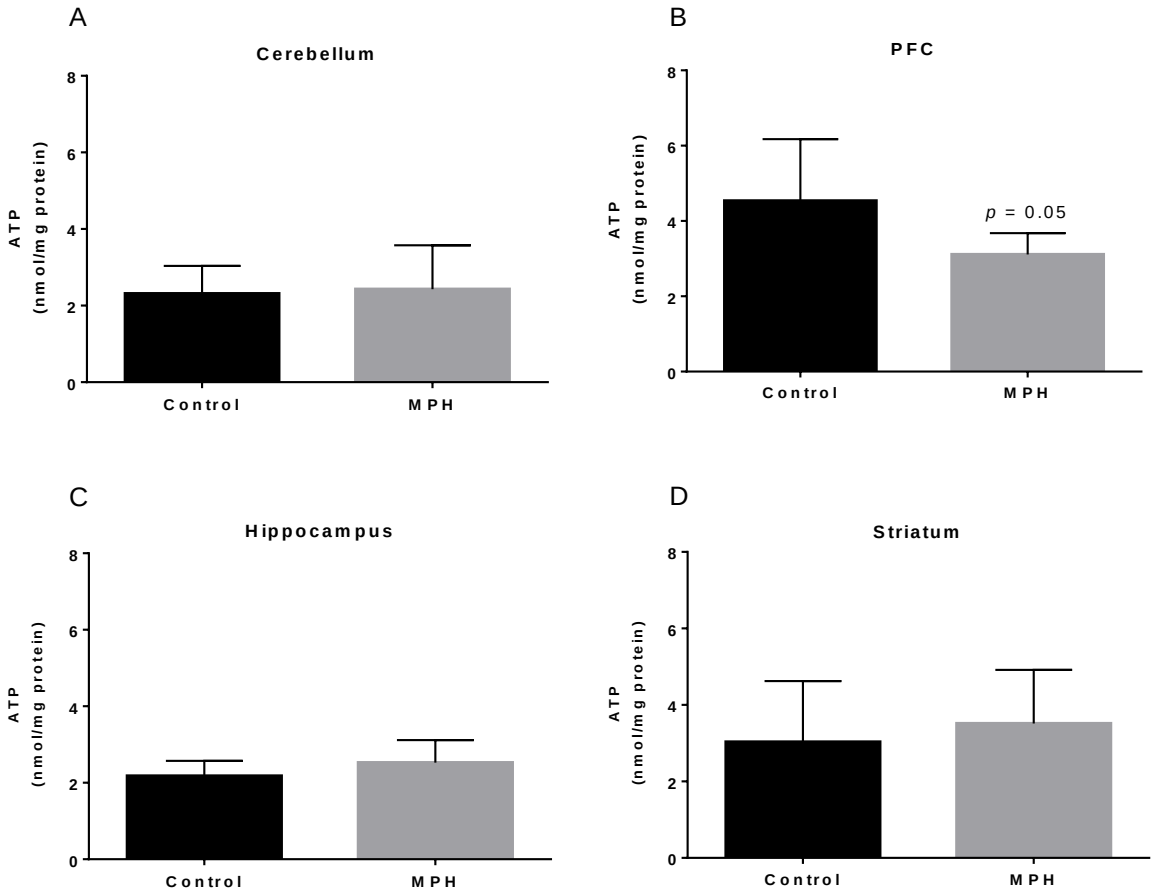

Figure 5 – ATP levels in the cerebellum (A), PFC (B), hippocampus (C), and striatum (D) of control and MPH-treated rats. Results, in nmol ATP/mg protein, from seven animals in each group are expressed as mean ± SD. Statistical analysis was made using the t-test.

4.5. MPH treatment reduced oxidative stress in the PFC and in the hippocampus

The levels of GSHt, GSSG, GSH, and the GSH/GSSG ratio in the PFC are presented in Figure 6. As observed, a significant increase in the GSH/GSSG ratio (p = 0.03) (Figure 6D) was found in rats treated with MPH. A strong tendency for a decrease (p = 0.050) in the GSSG levels was also detected. The remaining parameters in this area were not different among the two studied groups (Figures 6A, 6B, and 6C).

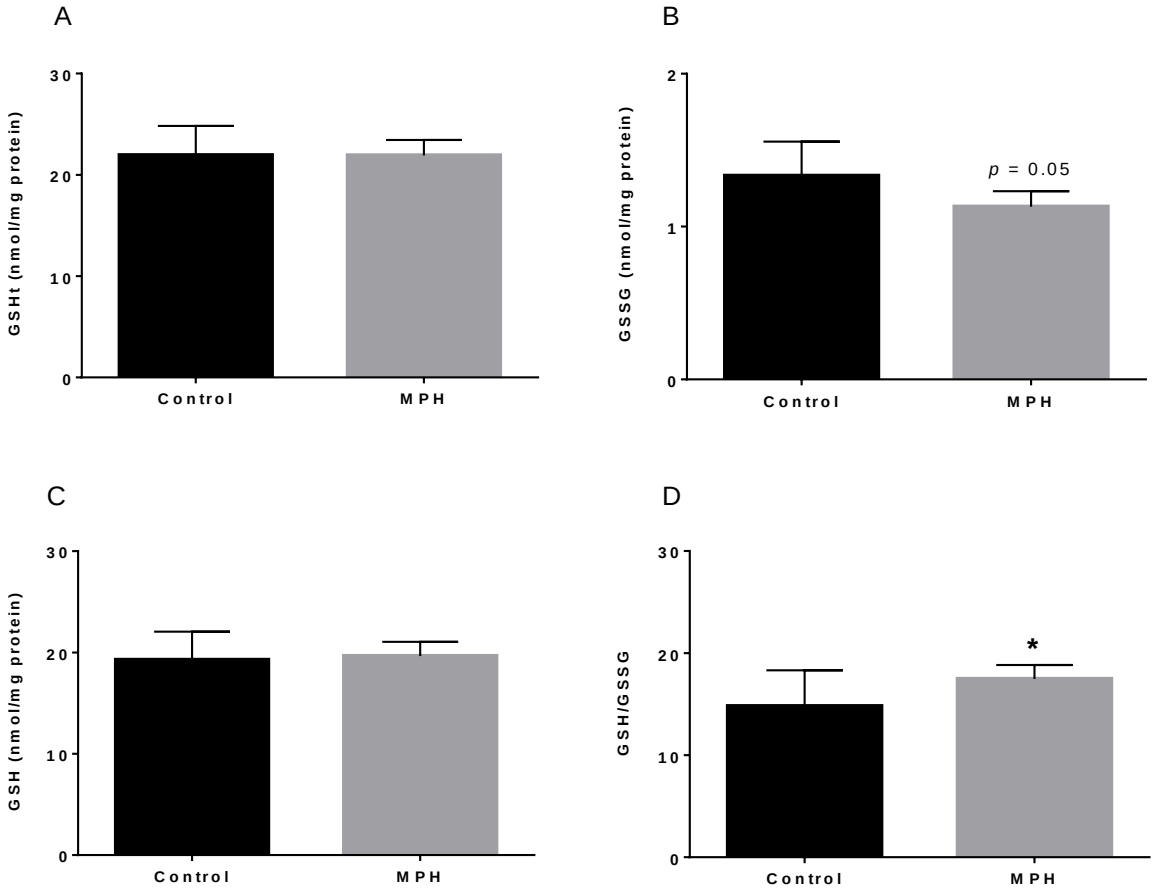

Figure 6 – Levels of GSHt (A), GSSG (B), GSH (C), and GSH/GSSG ratio (D) in the PFC of control and MPH-treated rats. Results, in nmol/mg protein or in GSH/GSSG ratio, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test for GSSG and GSH levels, and the Mann-Whitney Rank Sum test for GSHt and GSH/GSSG ratio levels ($^{*}p$<0.05 vs. control).

In Figure 7 are presented the levels of the four glutathione parameters evaluated in the hippocampus. MPH-treated rats showed a significant decrease of the levels of GSSG (p = 0.04) and a significant increase of the GSH/GSSG ratio (p = 0.006) (Figure 7B and

7D, respectively). No significant alterations were found in the levels of GSHt or GSH (Figure 7A and 7C, respectively) among both groups.

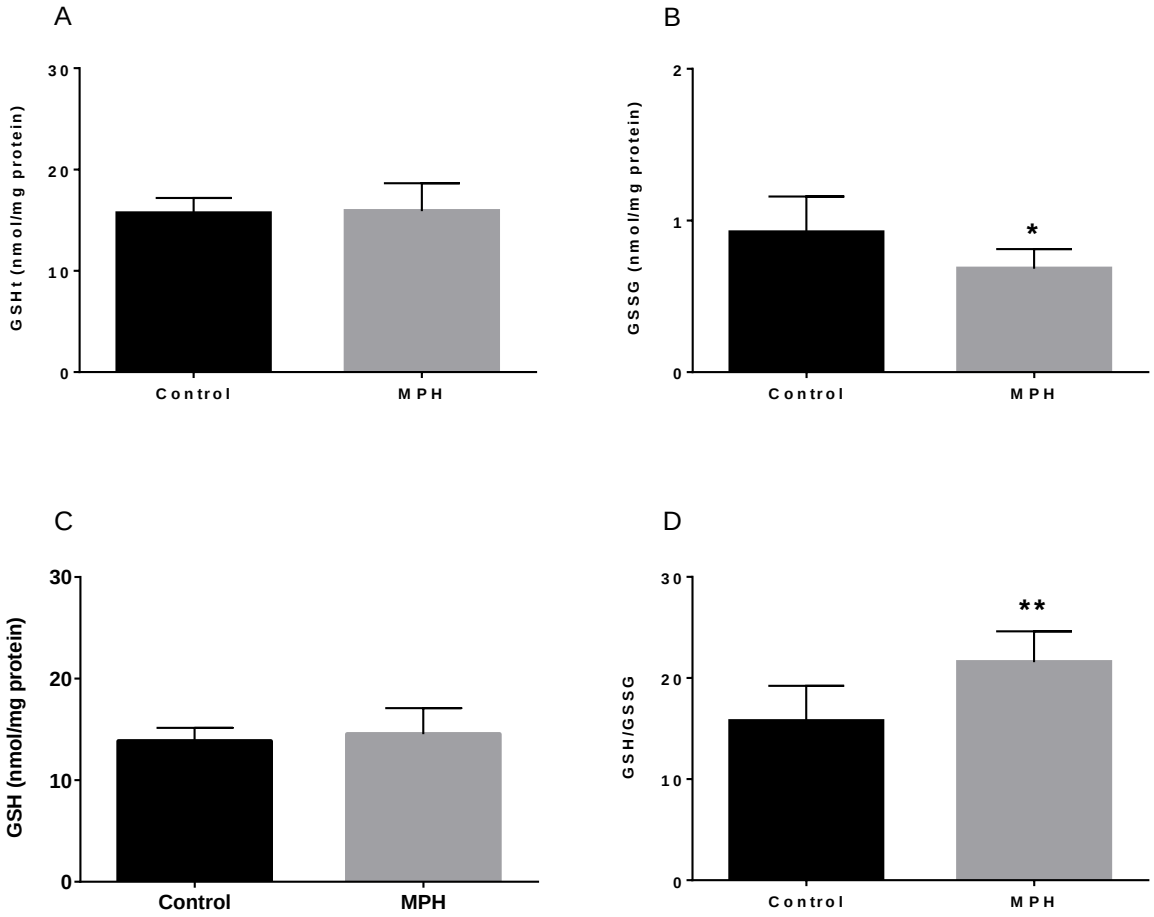

Figure 7 – Levels of GSHt (A), GSSG (B), GSH (C), and GSH/GSSG ratio (D) in the hippocampus of control and MPH-treated rats. Results, in nmol/mg protein or in GSH/GSSG ratio, are expressed as mean ± SD from seven animals of each group. Statistical analysis was made using the t-test ($^{*}p<0.05$ vs. control, $^{**}p<0.01$).

The levels of GSHt, GSSG, GSH, and GSH/GSSG ratio in the cerebellum and striatum are represented in Table 5. No significant differences were observed in these two referred brain areas among control and MPH-treated groups.

Table 5 – Glutathione parameters in the cerebellum and striatum of control and MPH-treated rats.

	Cerebellum		Striatum	
Parameter	**Control**	**MPH**	**Control**	**MPH**
GSHt (nmol/mg protein)	14.2 ± 0.8	14.3 ± 1.7	20.3 ± 6.4	22.9 ± 1.2
GSSG (nmol/mg protein)	0.8 ± 0.1	0.8 ± 0.2	1.0 ± 0.5	1.2 ± 0.4
GSH (nmol/mg protein)	12.6 ± 0.8	12.8 ± 1.5	18.4 ± 5.6	20.2 ± 1.3
GSH/GSSG (nmol/mg protein)	16.1 ± 2.4	17.1 ± 4.2	20.6 ± 5.1	17.3 ± 5.4

Data of GSHt, GSSG, and GSH levels, in nmol/mg protein and the GSH/GSSG ratio, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test for the GSHt, GSSG, GSH, and the GSH/GSSG ratio levels in the cerebellum, and GSSG and GSH levels in the striatum; the Mann-Whitney Rank Sum test was done for GSHt and the GSH/GSSG ratio levels in the striatum.

4.6. MPH treatment increased quinoprotein levels in the cerebellum

The quinoprotein levels in the cerebellum, PFC, hippocampus, and striatum are represented in Figure 8A, 8B, 8C, and 8D, respectively. Significant differences were found in the cerebellum (p = 0.03), since the MPH-treated rats revealed higher levels when were compared to the control animals (Figure 8A). Regarding the other three brain areas, no differences were found between the two groups (Figure 8B, 8C, and 8D).

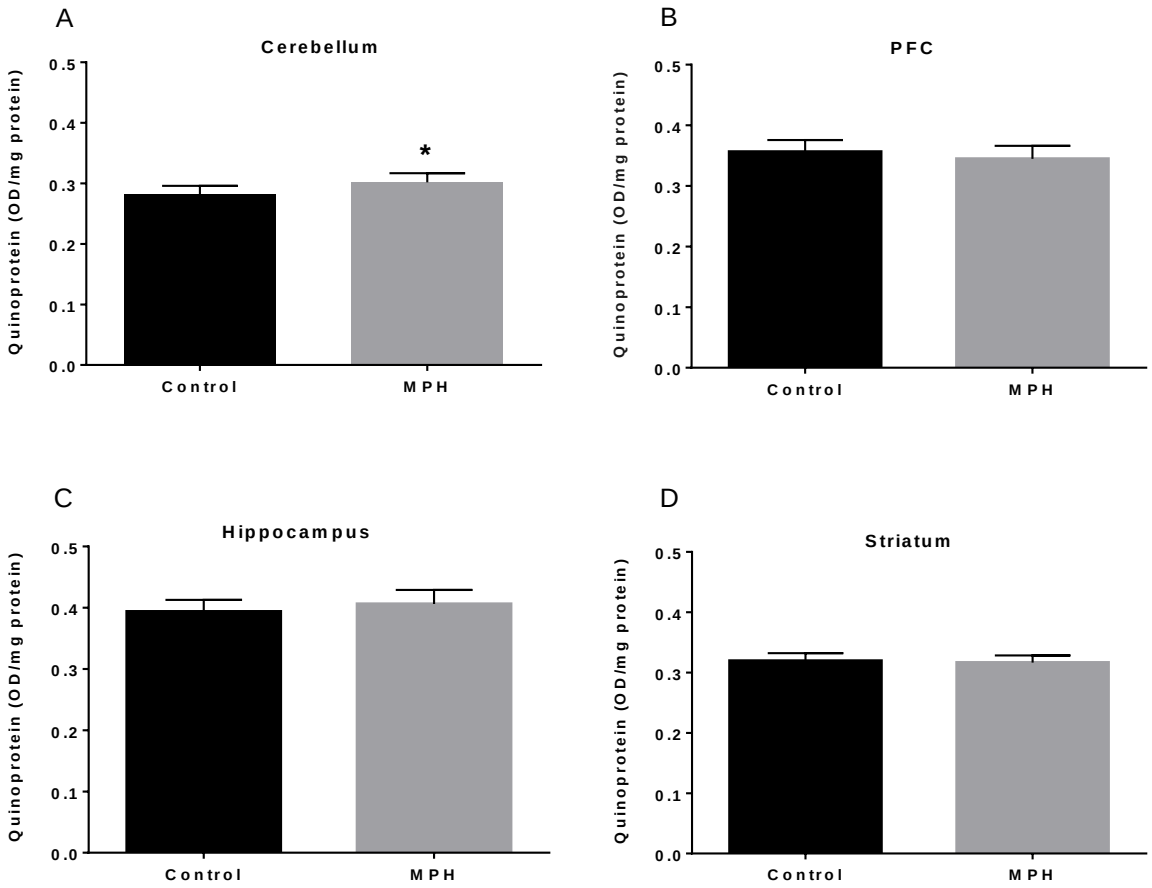

Figure 8 – Quinoprotein levels in the cerebellum (A), PFC (B), hippocampus (C), and striatum (D) of control and MPH-treated rats. Results, in OD/mg protein, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test (*$p < 0.05$ treatment vs. control).

4.7. Protein carbonylation was not affected by MPH treatment in the brain

The protein carbonylation levels were measured in the cerebellum, PFC, hippocampus, and striatum are presented in Table 6. No significant differences were observed among control and the MPH-treated rats in any of the four areas tested.

Table 6 – Protein carbonylation levels in cerebellum, PFC, hippocampus, and striatum of control and MPH-treated rats.

	Protein Carbonylation (% of control)	
Brain Areas	**Control**	**MPH**
Cerebellum	100 ± 26	103 ± 12
PFC	100 ± 18	93 ± 15
Hippocampus	100 ± 20	79 ± 19
Striatum	100 ± 26	111 ± 34

Results, in percent of control, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test.

4.8. ATP levels in the peripheral organs remained unchanged following MPH treatment

The ATP levels determined in the liver, heart, and kidneys showed no significant differences among the two studied groups, as can be seen in Table 7.

Table 7 – ATP levels in the liver, heart, and kidneys of control and MPH-treated rats.

	ATP (nmol/mg protein)	
Organ	**Control**	**MPH**
Liver	1.39 ± 1.20	1.74 ± 0.91
Heart	3.39 ± 2.01	2.19 ± 1.17
Kidneys	2.34 ± 1.36	1.33 ± 0.78

Results, in nmol ATP/mg protein, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test for the ATP levels in the liver and heart, and the Mann-Whitney Rank Sum test for the ATP values in the kidneys.

4.9. MPH treatment reduced GSSG levels in the heart

The redox status was evaluated in the heart and it is represented in Figure 9. As can be seen, GSSG levels were decreased (p = 0.049), GSH levels were increased (p = 0.04), and GSH/GSSG ratio was increased (p = 0.04). Regarding the GSHt levels, no significant differences were found among the two groups.

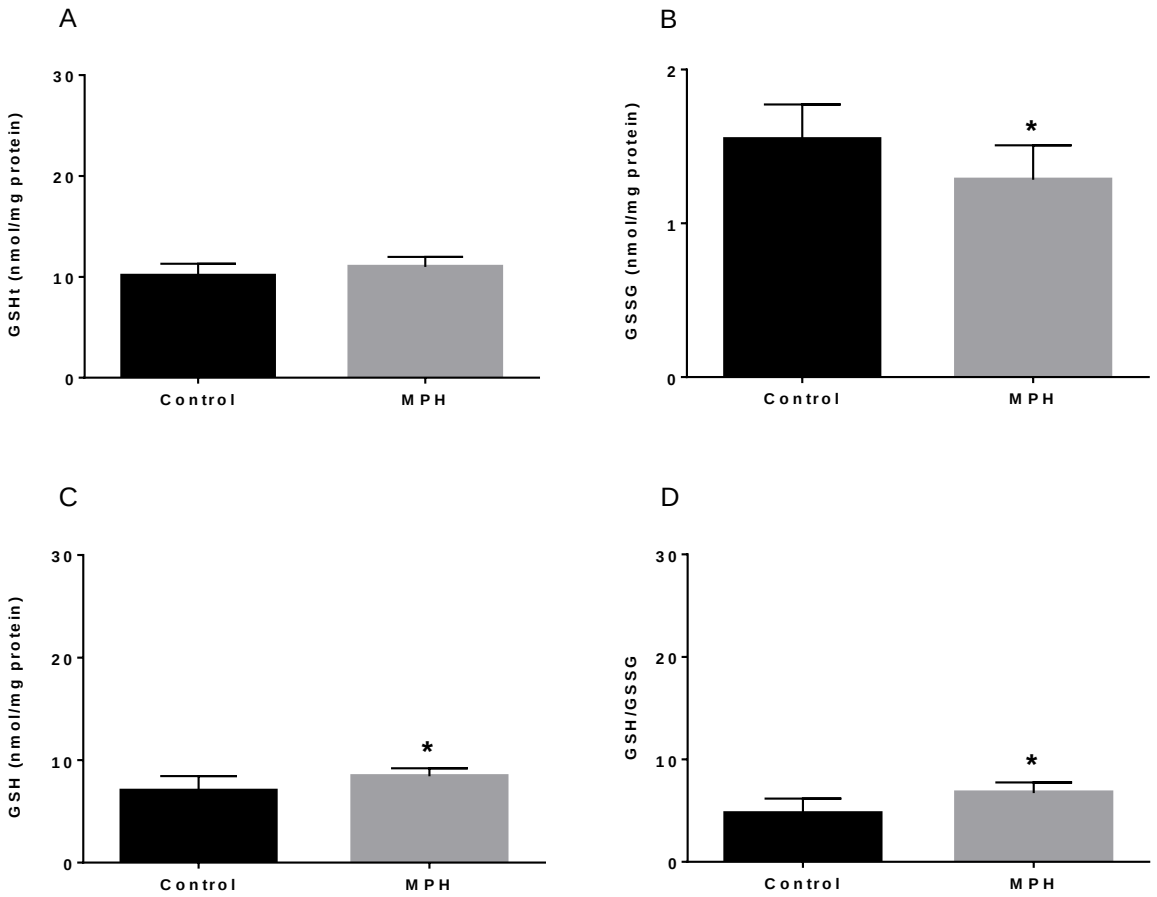

Figure 9 – Levels of GSHt (A), GSSG (B), GSH (C), and GSH/GSSG ratio (D) in the heart of control and MPH-treated rats. Results, in nmol/mg protein or in GSH/GSSG ratio, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test for GSHt, GSSG and GSH levels, and the Mann-Whitney Rank Sum test for the GSH/GSSG ratio levels ($^{*}p<0.05$ vs. control).

The levels of GSHt, GSSG, GSH, and GSH/GSSG ratio in the other two peripheral organs, liver and kidneys, are represented in Table 8. No significant differences among treated groups in either of the four parameters were observed in the liver or kidneys of the adolescent rats.

Table 8 – Glutathione parameters in the liver and kidneys of control and MPH-treated rats.

	Liver		Kidneys	
Parameter	**Control**	**MPH**	**Control**	**MPH**
GSHt (nmol/mg protein)	32.4 ± 3.9	33.3 ± 7.8	1.9 ± 0.9	2.1 ± 0.9
GSSG (nmol/mg protein)	5.2 ± 1.1	6.2 ± 1.4	0.2 ± 0.1	0.2 ± 1.1
GSH (nmol/mg protein)	21.9 ± 3.3	20.8 ± 5.9	1.6 ± 0.8	1.7 ± 0.8
GSH/GSSG (nmol/mg protein)	4.3 ± 1.0	3.4 ± 1.0	10.3 ± 2.8	9.1 ± 4.3

Data of GSHt, GSSG, and GSH levels, in nmol/mg protein or in GSH/GSSG ratio, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test for the GSHt, GSSG, GSH, and the GSH/GSSG ratio levels in the liver, and GSSG and the GSH/GSSG ratio levels in the kidneys. The Mann-Whitney Rank Sum test was used for the analysis of the GSHt and GSH levels in the kidneys.

4.10. Quinoprotein levels in the peripheral organs were not altered by MPH treatment

In Table 9 are represented the results of the quinoprotein levels in the liver, heart, and kidneys of adolescent rats after the exposure to 5 mg/kg of MPH or 5% sucrose for seven days. No significant differences were found in any of the peripheral organs.

Table 9 – Quinoproteins levels in the liver, heart, and kidneys of control and MPH-treated rats.

	Quinoproteins (OD/mg protein)	
Organ	**Control**	**MPH**
Liver	0.14 ± 0.01	0.15 ± 0.01
Heart	0.28 ± 0.02	0.27 ± 0.02
Kidneys	0.21 ± 0.04	0.18 ± 0.03

Results, in OD/mg protein, are expressed as mean ± SD from seven animals in each group. Statistical analysis was made using the t-test for the quinoprotein levels in the heart, and the Mann-Whitney Rank Sum test for the statistical analysis of the quinoprotein levels in the liver and kidneys.

4.11. Protein carbonylation levels in the peripheral organs remained unaltered following MPH treatment

Protein carbonylation levels in the liver, heart, and kidneys after the experimental protocol are presented in Table 10. No significant differences were observed in MPH-treated group when compared to the control group.

Table 10 – Protein carbonylation levels in the liver, heart, and kidneys of control and MPH-treated rats.

	Protein Carbonylation (% of control)	
Organ	**Control**	**MPH**
Liver	100 ± 19	118 ± 29
Heart	100 ± 36	86 ± 7
Kidneys	100 ± 36	104 ± 35

Results, in percent of control, of seven animals in each group are expressed as mean ± SD. Statistical analysis was made using the t-test for the levels of protein carbonylation in the heart, and the Mann-Whitney Rank Sum test for the levels of protein carbonylation in the liver and kidneys.

4.12. MPH promoted tissue changes in the peripheral organs, mainly promoting damage to the heart and kidneys

The qualitative histologic examination of peripheral organs (liver, heart, and kidneys) of control and MPH-treated rats was done using the haematoxylin and eosin staining (Figures 10, 11, and 12).

The liver of control animals showed a normal histological structure although a slight hepatocytes vacuolization surrounding the portal spaces was observed (Figure 10B and 10D). In MPH-treated rats, the hepatic structure was also preserved, however a moderate blood congestion and a more pronounced hepatocyte vacuolization in the periportal areas comparatively to controls was observed (Figure 10B and 10D). No necrotic zones or interstitial inflammatory cell infiltration was observed in either groups.

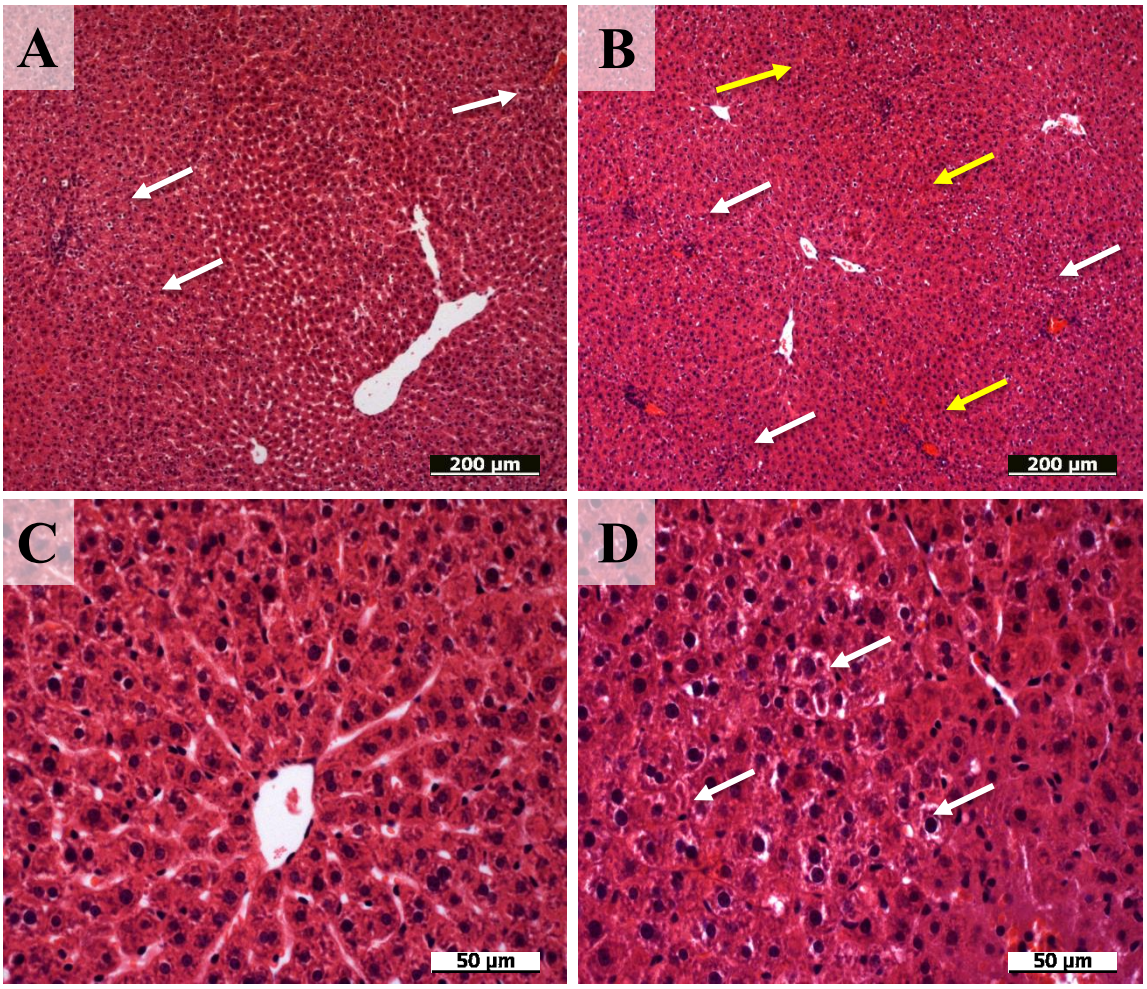

Figure 10 – Representative photomicrographs of liver sections stained with hematoxylin/eosin from control (A, C) and MPH-treated rats (B, D). A and C depict a normal histological structure, although with a slight micro vesicular vacuolization affecting the hepatocytes nearby the portal spaces (white arrows). However, this hepatocyte vacuolization was more notorious in MPH-treated animals (white arrows) as depicted in B and D; sign of blood congestion (yellow arrows) can also be observed in B.

In the heart, the control group showed a preserved tissue structure (Figure 11A and 11C). The MPH-treated animals showed an increase of the interstitial space, suggestive of oedema, with focal deposition of fibrin-like material, which were accompained by an intense vascular congestion (Figure 11B and 11D).

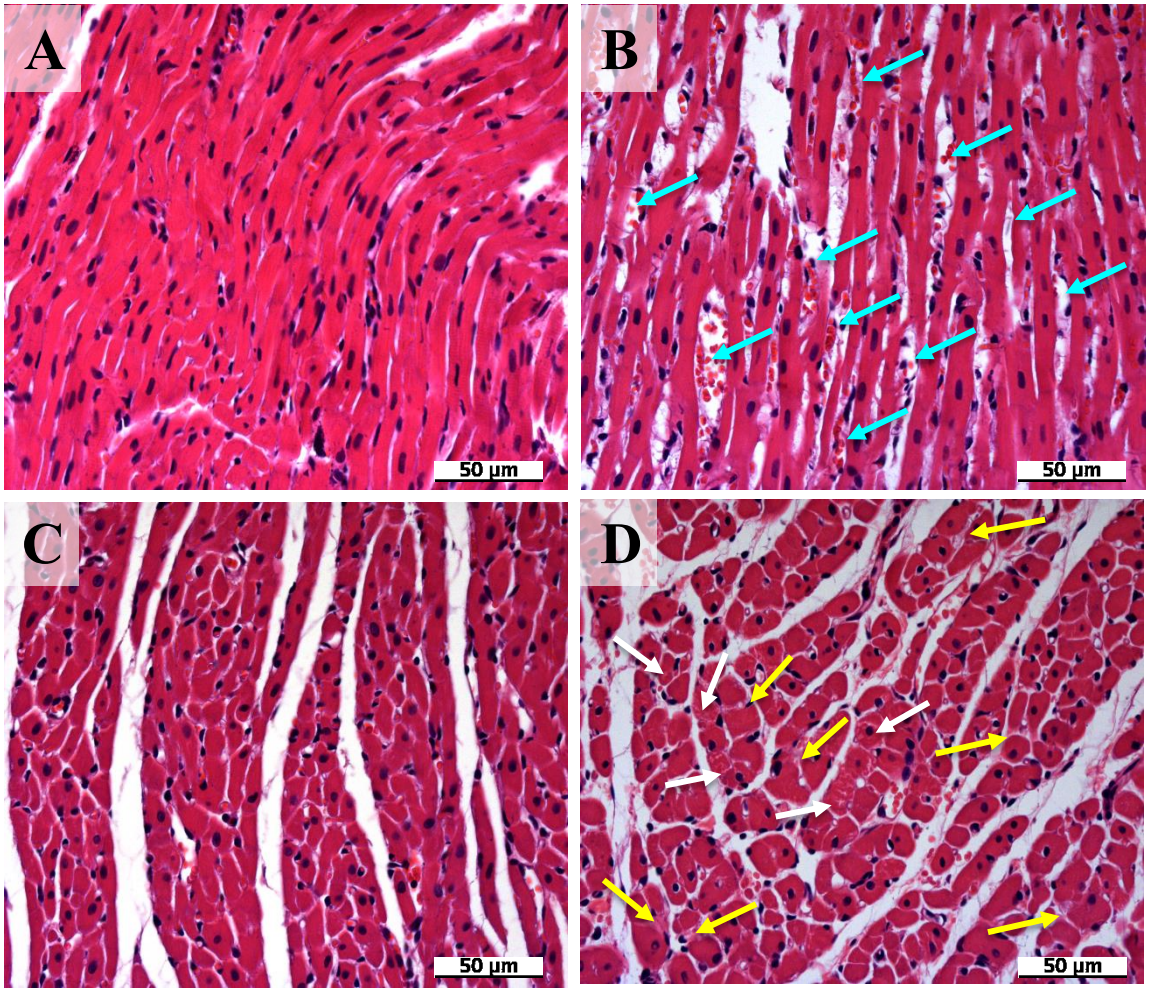

Figure 11 – Representative photomicrographs of heart sections from control (A, C) and MPH-treated rats (B, D) stained with hematoxylin and eosin. A and C show a normal histological structure. Signs of vascular congestion, with enlarged blood vessels filled with erythrocytes are depicted in B (blue arrows); in D, a general enlargement of interstitial space with focal deposition of fibrin-like material (yellow arrows) is observed; some cardiomyocytes with cytoplasmic vacuolization are also observed in D (white arrows).

The renal tissue organization remained well-maintained in the control group (Figure 12A and 12C). However, the MPH-treated group presented intense cellular damage, mainly affecting the proximal tubules, with cellular vacuolization, pyknotic nuclei, necrotic areas and leucocyte infiltrations (Figure 12B and 12D). Moreover, the glomeruli of these animals showed reduced Bowman's space with cellular infiltrations nearby the Bowman's capsule. The wall of the renal arteries was thicker in the MPH-treated animals with an apparent proliferation of smooth muscle cells (Figure 12B and 12D).

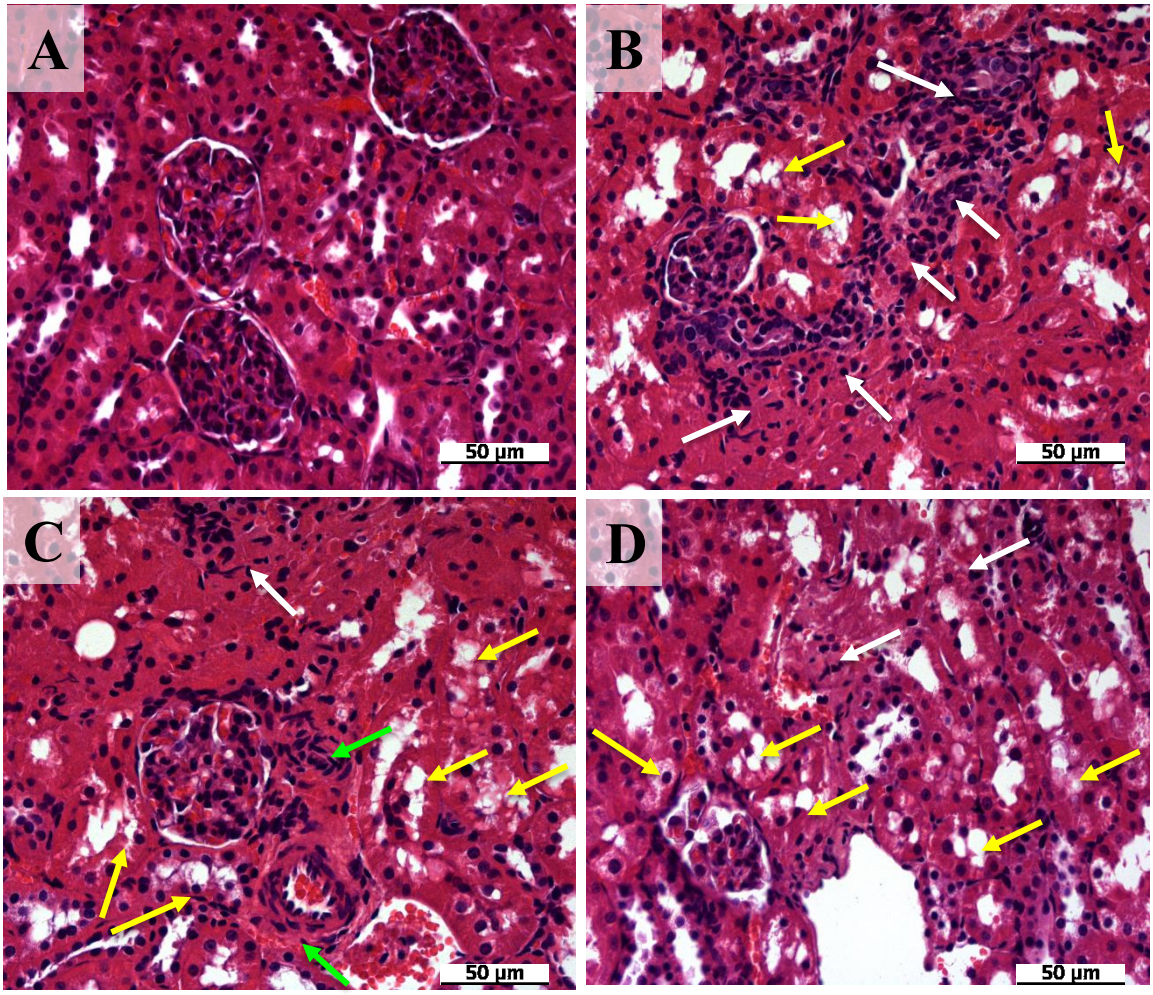

Figure 12 – Representative photomicrographs of kidney sections from control (A) and MPH-treated rats (B, C, D) stained with the hematoxylin and eosin. The renal structure was histologically preserved in A. Extensive necrotic zones with tissue disorganization and cellular infiltration (white arrows) as well as abundant sign of cellular vacuolization, mainly affecting the proximal tubes (yellow arrows), are depicted in B, C, and D. The greater thickness of arteriolar walls with an apparent proliferation of smooth muscle cells nearby the glomerulus (green arrows) and the reduction of Bowman's space are also depicted in C.

4.13. MPH treatment did not cause changes in fibrous tissue in the peripheral organs

The assessment of fibrous tissue in the liver, heart, and kidneys of both control and MPH-treated animals was done by the Picrosirius Red technique, which stains collagen red and muscle tissue yellow. The results are presented in Table 11 and no significant differences were observed among studied groups in the percentage of collagen/muscle area of the peripheral organs tested.

Table 11 – Collagen detection in the liver, heart, and kidneys of control and MPH-treated rats.

	Collagen (% collagen/muscle area)	
Organ	**Control**	**MPH**
Liver	0.80 ± 0.42	1.32 ± 0.69
Heart	1.98 ± 0.53	1.90 ± 0.44
Kidneys	2.04 ± 0.44	2.03 ± 0.45

Results, in percent collagen/muscle area, are expressed as mean ± SD from four animals in each group. Statistical analysis was made using the Mann-Whitney Rank Sum test.

Part V

Discussion and Conclusions

5. Discussion and Conclusions

MPH is the primary stimulant prescribed for the treatment of ADHD; however, its increasing use and abuse during critical stages of neurodevelopment in children and adolescents, has raised great concerns about possible consequences to the long-term exposure to MPH. Although preclinical studies in laboratory animals have been conducted over the years, the vast majority does not have into account two major factors with clinical relevance: the use of pharmacologically relevant doses and the route of administration. Clinical use of MPH typically involves oral administration of relatively low doses, whereas, the majority of the studies tend to use high doses, generally administrated via i.v., i.p., or subcutaneously. Moreover, some studies use adult animals, while the MPH use begins mainly in childhood. Therefore, in the present study, we studied the effects of a clinical MPH dose scheme (2 x 5 mg/kg, 5h apart, oral route) in an adolescent rat model for seven days. A brief discussion of the major findings follows.

5.1. MPH did not promote temperature, weight or food/water intake changes

Hyperthermia is one of the most lifethreatening effects among stimulant drugs. The increase of the CNS metabolic activity can lead to a dysregulation between heat production and dissipation with the drug use (Carvalho et al., 2012). In fact, 3,4-methylenedioxymethamphetamine (MDMA), an amphetamine derivate commonly used in recreational scenarios, is one of the most studied amphetamine regarding its hyperthermic effects, since elevated body temperature after exposure to this drug has been reported in both animals and humans following similar conditions of exposure (i.e., same dose scheme and route of administration) (Carvalho et al., 2012, Teixeira-Gomes et al., 2016). Since MPH is also a stimulant with a similar pharmacology to AMPH, the body temperature of the rats in several time-points throughout the experimental period was measured. However, no significant differences were found between control and MPH-treated groups. This result contrasts to early findings where a significant hyperthermia (41.5ºC) was reported 2 h after the fourth injection of MPH (4 x 22 mg/kg, every 2 h, i.p.) in male Sprague Dawley rats with PND 83 to 87 by Levi and coworkers (Levi et al., 2012). In the same study, they also concluded that the threshold dose of MPH necessary to significantly raise the body temperature in rats was 10-fold higher than the one necessary for AMPH exposure. Accordingly, hyperthermia is somewhat less expected to happen after overdoses of MPH than with AMPH (Levi et al., 2012). In fact, hyperthermia is a rare

acute effect that has been reported only after severe intoxications of MPH and not after ingestion of therapeutic doses (Scharman et al., 2007, Peyre and Delorme, 2012). Therefore, our low dose scheme, comparable to the human situation, can explain why the body temperature of adolescent rats was not changed by MPH treatment.

In addition to hyperthermia, we also evaluated body weight gain and food/water intake in adolescent rats during the seven days treatment. In the literature, significant differences were found in appetite, food intake, and, consequently, in weight gain in rodents and humans treated with MPH (Rapport and Moffitt, 2002, Gray et al., 2007, Alam and Najam, 2015, Thanos et al., 2015). A tendency for decrease in body weight gain was found in the MPH-treated group in our study, though not statistically significant. The same tendency was also reported by Alam and Najam. After the oral administration of MPH twice a day (5mg/kg/day) for 4 weeks, a tendency for weight loss in the third week was observed (Alam and Najam, 2015). Significant decreases were also found with a lower (2 mg/kg/day) and a higher dose (8 mg/kg/day) in the same regime, which relates the long-term administration to the consequent weight loss (Alam and Najam, 2015). In another study, the weight loss was also reported after rats aged 4 weeks received a dual dosage of 4 mg/kg and 10 mg/kg or 30 mg/kg and 60 mg/kg of MPH administered in daily drinking water for three months. The lower dose was administrated in the first hour and the higher dose was administrated for seven hours (Thanos et al., 2015). These previous two studies used either higher doses and/or longer times of drug treatment, which may explain the different findings in weight gain to our study. Regarding the food and water intake, the published data is contradictory. In our study, MPH treatment did not affect both food and water intake; however, Alam and Najam reported an increased food and water intake in all weeks and at all doses tested (2, 5, or 8 mg/kg/day) (Alam and Najam, 2015). Meanwhile, Thanos and coworkers reported a decreased food intake particularly in the first five weeks of a three months treatment (Thanos et al., 2015). Overall, our results show that MPH is somehow related with weight loss but that loss is not directly related to food/water intake. Hence, there is a need to understand the mechanisms underlying this effect since decreased appetite, weight loss and anorexia are reported as side effects of MPH in humans (Rapport and Moffitt, 2002).

5.2. MPH induced brain changes regarding energetic content, redox status and quinoprotein formation

The potential neurotoxicity of MPH has raised attention in the last years but there are still few studies concerning the effects of a long time exposure to this stimulant and the implications to the brain. Thus, we aimed to verify if a pharmacologically relevant MPH

regimen can cause significant changes in several brain areas, namely regarding ATP levels and oxidative stress.

In our study, no differences were found in the ATP levels in the cerebellum, hippocampus, and striatum of the MPH-treated adolescent rats; however, data showed a strong tendency for a decrease in ATP levels in the PFC. Interestingly, also in PND 40 Wistar rats, our group found a significant decrease in the ATP content of the frontal cortex following three MDMA doses (each dose 5 mg/kg, i.p., every 2 h), but not in the cerebellum, hippocampus, and striatum (Teixeira-Gomes et al., 2016). To the best of our knowledge, no previous studies *in vivo* evaluated this parameter after MPH exposure. Published data for MPH focused on the activity of the mitochondrial respiratory chain (Fagundes et al., 2010a) and the activity of enzymes involved in the Krebs cycle (Reus et al., 2013). A study with young Wistar rats (PND 25), which received a single injection of MPH (1, 2 or 10 mg/kg, via i.p.) or an injection once a day for 28 days, showed a reduction in the activity of complex I in the cerebellum and PFC after the acute treatment, and an increase in the activities of complexes II and IV after the chronic treatment (Fagundes et al., 2010a). Another study reported a decrease activity on citrate synthase and isocitrate dehydrogenase in the cerebellum, striatum, PFC, hippocampus, and cortex after young Wistar rats (PND 25) received MPH (1.0, 2.0 or 10 mg/kg, via i.p.) during 28 days, while, after a single injection, the activity of both enzymes was not altered in any of the brain areas studied (Reus et al., 2013). Of note, mitochondria generate most of the energy of the brain by converting oxygen and nutrients into ATP, so, if any abnormality is detected in these organelles, the neuronal function and survival can be affected since ATP is critical for the correct functioning of the brain (Barbosa et al., 2015). When comparing the studies previously done with our results, it can be seen that the animals' age, the dosage regimen, the time of exposure, and the route of administration are important factors regarding the energetic impairment in the brain after MPH exposure. Still, our work raises the question about the ATP levels in the brain of adolescent rats after a dose scheme that reproduces the human scenario, and, it is possible to speculate that MPH may promote energetic impairment in the cortex, as did MDMA in animals with the same age. A more profound investigation is needed to better understand this phenomenon.

The assessment of oxidative stress in the brain after exposure to MPH was also done. Earlier evidence already reported increased lipid peroxidation in several brain areas after different MPH dose schemes (Martins et al., 2006, Schmitz et al., 2012, Motaghinejad et al., 2016). Additionally, it is known that oxidative stress plays an important role in amphetamines neurotoxicity (Carvalho et al., 2012), so, since MPH is equally a stimulant, we determined oxidative stress parameters like the glutathione levels,

quinoprotein formation, and protein carbonylation in the cerebellum, PFC, hippocampus, and striatum of adolescent rats exposed for one week to MPH.

Our findings showed that MPH treatment increased the antioxidant status of the PFC and hippocampus, as this work found increased GSH/GSSG ratio in the PFC and in the hippocampus, whereas decreased GSSG levels in the later area. No differences were found in the glutathione levels in the cerebellum and striatum. To the best of our knowledge, this was the first report of the glutathione status in adolescent rats after one-week exposure to MPH (2 x 5 mg/kg, 5 h apart, oral route). Of note, GSH is the most important cellular antioxidant and it is critical for the detoxification of xenobiotics and their metabolites and to maintain the intracellular redox balance (Owen and Butterfield, 2010). Under normal conditions, GSH is the most prevalent form, however, under oxidative stress, GSSG levels increase, so, the GSH/GSSG ratio has been used as an index of oxidative stress (Costa et al., 2011). In our study, the GSH/GSSG ratio increased in the PFC and the hippocampus, so, this is the first experimental demonstration that MPH reduces oxidative stress in the brain of adolescent rats after the administration of therapeutic relevant doses. Recently, a preclinical study reported a dose-dependent increase of GSSG content and a reduction of GSH content in the hippocampus of Wistar rats with 8 weeks old after chronic treatment (21 days) with high doses of MPH (2.0, 5.0, 10, or 20 mg/kg, via i.p.) (Motaghinejad et al., 2016). However, the age of the animals and the duration of the protocol seem the most relevant differences to explain that induced oxidative stress.

Regarding the protein-bound quinone formation, a significant increase in the cerebellum was observed in the MPH-treated group. No differences were observed in the other brain areas evaluated. Once again, no previous studies were found that evaluate this parameter after MPH administration. Nevertheless, quinones can promote the formation of ROS and, consequently, induce severe oxidative stress. ROS can bind to macromolecules like lipids, proteins, and DNA (Bolton et al., 2000). It remains to be clarified why the cerebellum is the only brain area affected.

Another oxidative stress parameter evaluated was the protein carbonylation, an irreversible oxidative damage marker to proteins associated to a permanent loss of cellular function that can lead to tissue damage (Dalle-Donne et al., 2006). Briefly, carbonyl groups are formed in proteins by the reaction of ROS and the protein or by direct oxidation of proteins by ROS. If the carbonylated proteins are not degraded, their accumulation leads to cell and tissue injury (Dalle-Donne et al., 2006). Recently, studies showed that MPH induces the protein carbonyl formation in specific brain areas (Martins et al., 2006, Comim et al., 2014). Martins and coworkers reported a dose-dependent increase on protein carbonylation in the cerebellum, PFC, hippocampus, and striatum of

young (PND 25) and adult Wistar rats (PND 60) after i.p. injections of MPH (1.0, 2.0, or 10 mg/kg), once daily, during 28 days (Martins et al., 2006). Another study reported protein damage in the cerebellum and hippocampus of adult SHR rats (PND 60) after chronic exposure to MPH (2 mg/kg, via i.p., once daily) during 28 days (Comim et al., 2014). In our study, no significant differences were observed between the groups in any of the four areas tested. When comparing the studies, it can be seen that they differ in several factors that have impact in the pharmokinetic profile of MPH and, consequently, in the results. Among the factors, we highlight the animals' age, the animal model (Wistar rat vs. SHR rat, the animal model of ADHD), the dosage regimen and long treatment period, and the i.p. route of administration. Overall, it became demonstrated that MPH was not able to increase protein carbonylation in the brain of adolescent rats after one-week exposure to a pharmacological relevant oral dose.

5.3. MPH induced organs-related changes regarding redox status and cellular damage

Case reports of liver, heart, and kidneys injuries attributed to MPH are found in the literature but there are very few studies in animals. Therefore, it is of extreme importance to investigate the effects of MPH on peripheral organs of adolescent animals and try to understand if therapeutic doses could promote any histological or functional adverse effect as it was done in the present work.

As biomarkers of liver or heart integrity, plasma ALT, AST, CK-MB and total-CK were measured and no differences were found between the control and the MPH-treated group.

When we evaluated the ATP levels, no differences were found in the liver, heart, or kidneys of MPH-treated rats when compared to the control group. In the literature, no *in vivo* or *in vitro* studies reporting the ATP levels in the peripheral organs of laboratory animals after MPH exposure were found. Thus, to the best of our knowledge, our work gives the first hint that therapeutic doses of MPH do not impair the energetic content of the peripheral organs evaluated.

Regarding the oxidative stress parameters, a reduction of the GSSG levels, an increase of the GSH levels, and, consequently, an increase of the GSH/GSSG ratio were observed only in the heart. As mentioned above, the GSH/GSSG ratio is as an index of oxidative stress and, since an increase of this ratio was observed in the heart, we can speculate that MPH increases the cardiac antioxidant status. No differences were observed in the glutathione levels of the liver and the kidneys following MPH treatment.

Additionally, the peripheral organs did not presented any alteration in the other two oxidative stress parameters evaluated, quinoprotein levels and protein carbonylation. To the best of our knowledge, the oxidative stress was, for the first time, evaluated in the peripheral organs of adolescent rats exposed to MPH. Altogether, these results indicate that oxidative stress does not occur in the peripheral organs of adolescent rat following therapeutic oral doses of MPH. Moreover, therapeutic oral doses might even provide protection to the heart against oxidative stress, according to our data.

The histological examination of each peripheral organ provided us more information about the possible toxic effect of MPH. In the liver of MPH-treated rats, we found signals of blood congestion and a more pronounced hepatocyte vacuolization in the periportal areas. No necrotic zones or interstitial inflammatory cell infiltration was observed in either group. While MPH did not induced any significant hepatotoxicity under our experimental protocol, another study reported hepatocellular necrosis 24 h after an i.v. administration of MPH (100 mg/kg) to ICR male mice (Roberts et al., 1994), which corroborates the reported liver injury due i.v. abuse of MPH in humans (Mehta et al., 1984, Stecyk et al., 1985). However, the dose described in that study (100 mg/kg) is very high and might correspond to an abuse scenario, unlike our oral dose scheme that corresponds to a clinical setting.

In the heart, a general enlargement of the interstitial space with focal deposition of fibrin-like material and an intense vascular congestion was observed. As previously mentioned in section 1.4.2.1., pronounced lesions in the myocardium were associated to MPH administration in two laboratory animals models after a different dose regimen (1) Swiss-Webster mice received 2.5 or 5.0 mg/kg of MPH, three times a week, i.p., or, 5.0 mg/kg, once a day, via oral, during 4 or 14 weeks; (2) Sprague Dawley rats received 2.0, 20, or 100 mg/kg of MPH, via i.p., for periods of 3, 6, or 9 weeks (Henderson and Fischer, 1995). Additionally, myofibril structure disruption related with mitochondria degeneration was found after oral administration of MPH (5.0, 10, or 20 mg/kg) to PND 25 Wistar rats for 5 days/week during three months (Take et al., 2008). Although the animals' age, the dose regimen, the administration route, and the time of exposure were different in the three studies, MPH has the potential to damage the heart even after therapeutic doses given to adolescent rats. These data corroborate the cardiotoxic events attributed to this stimulant in humans either after long-term treatment with therapeutic doses or following overdoses (Lucas et al., 1986, Daly et al., 2008, Nymark et al., 2008, Thompson and Thompson, 2010, Munk et al., 2015). Regarding the mechanism behind these cardiotoxicity, more studies are required, but oxidative stress should be not involved. In fact, an increase in the antioxidant status of the heart was found. Possibly, vascular changes promoted by MPH can be the missing link.

Regarding the renal tissue, the MPH-treated group presented extensive necrotic zones, cellular infiltration, and cellular vacuolization mainly affecting the proximal tubes. It was only possible to find one other study focused on the possible renal effects of MPH by performing both *in vivo* and *in vitro* assays (Salviano et al., 2015). Twenty-four hours after oral administration of MPH (10 mg/kg) to adult male Wistar rats, the kidneys of both control and treated groups showed a preserved tissue structure. Despite this, *in vitro* studies showed that the addition of 10 µg/ml of MPH to the isolated rat kidney significantly reduced the glomerular filtration rate, the urine flow, and the sodium transport (Salviano et al., 2015). MPH may have a nephrotoxic effect depending on the time of exposure, but more studies have to be made to better understand what mechanisms are implied in the MPH-induced injury kidney. However, it seems clear that oxidative stress does not play a role in these toxic events to the kidney, since no differences were found in oxidative stress parameters in this organ.

The histological examination corroborates the results obtained by the plasma ALT, AST, CK-MB, and total-CK since there were no signs of necrosis in any of the peripheral organs. Additionally, the conjunctive tissue ratio was also assessed in our study, as putative index of drug-induced injury. Briefly, an accumulation of conjunctive tissue leads to fibrosis, and may be the result of stimuli like drug exposure, persistent infections, and autoimmune reactions (Wynn, 2008). The fibrotic cascade is mediated by complex biological processes and, as it progresses, impairments in the tissue architecture and function and, eventually, organ failure can occur (Ueha et al., 2012). In our work, the amount of fibrous tissue was not altered by MPH treatment. Despite this, we found that MPH may induce heart and kidneys injuries in adolescent rats, but it seems that vascular responses promoted by MPH might be the determinants for that injury since fibrosis is the end result of chronic inflammation and was not verified in our study (Wynn, 2008, Ueha et al., 2012). Importantly, our exposure period to MPH was possibly short, unabling this kind of damage to occur. So, more studies are necessary to confirm if MPH induces fibrosis in peripheral organs.

5.4. Conclusions

The worldwide rise in ADHD diagnosis and, consequently, the widespread prescription of MPH has raised substantial debates regarding the long-term effects resulting from an early exposure to this drug. In this dissertation, we showed that MPH increased the GSH/GSSG ratio in the PFC and hippocampus, which suggests that, at therapeutic doses orally administrated to adolescent rats, this drug increases antioxidant

defenses and might have a protective role in these two brain areas. On the other hand, MPH exposure in adolescent rats did not promote any significant changes in ATP content or protein carbonylation in the brain areas, but, significantly increased the quinoprotein levels in the cerebellum. Regarding the peripheral organs, the MPH dose scheme used did not alter the ATP, quinoprotein, or protein carbonylation levels in any of the organs studied. However, once again, MPH showed a protective role in the heart of MPH-treated rats, since the GSSG levels were significantly reduced in this organ. Interestingly, MPH compromised the normal tissue organization mainly in the heart and kidneys without promoting any changes in the plasma biomarkers of liver or heart integrity and in the amount of fibrous tissue.

This study showed for the first time that therapeutic oral doses of MPH are might have a beneficial role in the levels of glutathione in adolescent rats in the brain and heart, though they might promote several tissue morphologic changes, mainly vascular related changes, in the heart and kidney. Thus, further investigation is needed in order to understand the mechanisms behind these alterations and what factors can promote them.

Part VI

References

6. References

Alam N, Najam R (2015) Effect of repeated oral therapeutic doses of methylphenidate on food intake and growth rate in rats. Pak J Pharm Sci 28:9-13.

Aoyama T, Kotaki H, Iga T (1990) Dose-dependent kinetics of methylphenidate enantiomers after oral administration of racemic methylphenidate to rats. J Pharmacobiodyn 13:647-652.

Aoyama T, Kotaki H, Sasaki T, Sawada Y, Honda Y, Iga T (1993) Nonlinear kinetics of threo-methylphenidate enantiomers in a patient with narcolepsy and in healthy volunteers. Eur J Clin Pharmacol 44:79-84.

APA (1968) Diagnostic and Statistical Manual of Mental Disorders (DSM-II): American Psychiatric Association.

APA (1980) Diagnostic and Statistical Manual of Mental Disorders (DSM-III): American Psychiatric Association.

APA (1987) Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R): American Psychiatric Association.

APA (1994) Diagnostic and Statistical Manual of Mental Disorders (DSM-IV): American Psychiatric Association.

APA (2013) Diagnostic and Statistical Manual of Mental Disorders (DSM-V): American Psychiatric Association.

Arnsten AF (2010) The use of α-2A adrenergic agonists for the treatment of attention-deficit/hyperactivity disorder. Expert Rev Neurother 10:1595-1605.

Arnsten AF, Berridge CW, McCracken JT (2009) The neurobiological basis of attention-deficit/hyperactivity disorder. Primary Psychiatry 16:47-54.

Arnsten AF, Li BM (2005) Neurobiology of executive functions: catecholamine influences on prefrontal cortical functions. Biol Psychiatry 57:1377-1384.

Arnsten AF, Pliszka SR (2011) Catecholamine influences on prefrontal cortical function: relevance to treatment of attention deficit/hyperactivity disorder and related disorders. Pharmacol Biochem Behav 99:211-216.

Banihabib N, Es.Haghi M, Zare S, Farrokhi F (2014) The Effect of Oral Administration of Methylphenidate on Hippocampal Tissue in Adult Male Rats. Neurosurg Q.

Barbosa DJ, Capela JP, Feio-Azevedo R, Teixeira-Gomes A, Bastos ML, Carvalho F (2015) Mitochondria: key players in the neurotoxic effects of amphetamines. Arch Toxicol 89:1695-1725.

Barceloux DG (2012) Medical toxicology of drug abuse: synthesized chemicals and psychoactive plants: John Wiley & Sons, Inc.

Bethancourt JA, Camarena ZZ, Britton GB (2009) Exposure to oral methylphenidate from adolescence through young adulthood produces transient effects on hippocampal-sensitive memory in rats. Behav Brain Res 202:50-57.

Bidwell LC, McClernon FJ, Kollins SH (2011) Cognitive enhancers for the treatment of ADHD. Pharmacol Biochem Behav 99:262-274.

Bolanos CA, Barrot M, Berton O, Wallace-Black D, Nestler EJ (2003) Methylphenidate treatment during pre- and periadolescence alters behavioral responses to emotional stimuli at adulthood. Biol Psychiatry 54:1317-1329.

Bolea-Alamanac B, Nutt DJ, Adamou M, Asherson P, Bazire S, Coghill D, Heal D, Muller U, Nash J, Santosh P, Sayal K, Sonuga-Barke E, Young SJ, British Association for P (2014) Evidence-based guidelines for the pharmacological management of attention deficit hyperactivity disorder: update on recommendations from the British Association for Psychopharmacology. J Psychopharmacol 28:179-203.

Bolton JL, Trush MA, Penning TM, Dryhurst G, Monks TJ (2000) Role of quinones in toxicology. Chem Res Toxicol 13:135-160.

Brophy K, Hawi Z, Kirley A, Fitzgerald M, Gill M (2002) Synaptosomal-associated protein 25 (SNAP-25) and attention deficit hyperactivity disorder (ADHD): evidence of linkage and association in the Irish population. Mol Psychiatry 7:913-917.

Brunner D, Hen R (1997) Insights into the neurobiology of impulsive behavior from serotonin receptor knockout mice. Ann N Y Acad Sci 836:81-105.

Bruxel EM, Akutagava-Martins GC, Salatino-Oliveira A, Contini V, Kieling C, Hutz MH, Rohde LA (2014) ADHD pharmacogenetics across the life cycle: New findings and perspectives. Am J Med Genet B Neuropsychiatr Genet 165B:263-282.

Budur K, Mathews M, Adetunji B, Mathews M, Mahmud J (2005) Non-stimulant treatment for attention deficit hyperactivity disorder. Psychiatry (Edgmont) 2:44-48.

Bymaster FP, Katner JS, Nelson DL, Hemrick-Luecke SK, Threlkeld PG, Heiligenstein JH, Morin SM, Gehlert DR, Perry KW (2002) Atomoxetine increases extracellular levels of norepinephrine and dopamine in prefrontal cortex of rat: a potential mechanism for efficacy in attention deficit/hyperactivity disorder. Neuropsychopharmacology 27:699-711.

Cairns R, Daniels B, Wood DA, Brett J (2016) ADHD medication overdose and misuse: the NSW Poisons Information Centre experience, 2004-2014. Med J Aust 204:154.

Capela JP, Macedo C, Branco PS, Ferreira LM, Lobo AM, Fernandes E, Remiao F, Bastos ML, Dirnagl U, Meisel A, Carvalho F (2007) Neurotoxicity mechanisms of thioether ecstasy metabolites. Neuroscience 146:1743-1757.

Carlezon WA, Jr., Mague SD, Andersen SL (2003) Enduring behavioral effects of early exposure to methylphenidate in rats. Biol Psychiatry 54:1330-1337.

Carvalho M, Carmo H, Costa VM, Capela JP, Pontes H, Remiao F, Carvalho F, Bastos Mde L (2012) Toxicity of amphetamines: an update. Arch Toxicol 86:1167-1231.

Castellanos FX, Lee PP, Sharp W, Jeffries NO, Greenstein DK, Clasen LS, Blumenthal JD, James RS, Ebens CL, Walter JM, Zijdenbos A, Evans AC, Giedd JN, Rapoport JL (2002) Developmental trajectories of brain volume abnormalities in children and adolescents with attention-deficit/hyperactivity disorder. JAMA 288:1740-1748.

Cavallotti C, Nuti F, Bruzzone P, Mancone M (2002) Age-related changes in dopamine D2 receptors in rat heart and coronary vessels. Clin Exp Pharmacol Physiol 29:412-418.

Chai G, Governale L, McMahon AW, Trinidad JP, Staffa J, Murphy D (2012) Trends of outpatient prescription drug utilization in US children, 2002-2010. Pediatrics 130:23-31.

Challman TD, Lipsky JJ (2000) Methylphenidate: its pharmacology and uses. Mayo Clin Proc 75:711-721.

Chan YP, Swanson JM, Soldin SS, Thiessen JJ, Macleod SM, Logan W (1983) Methylphenidate hydrochloride given with or before breakfast: II. Effects on plasma concentration of methylphenidate and ritalinic acid. Pediatrics 72:56-59.

Charatan F (2006) FDA committee votes for warning labels on stimulant drugs. BMJ 332:380.

Clements SD (1966) Minimal brain dysfunction in children: terminology and identification: phase one of a three-phase project: US Department of Health, Education and Welfare.

Comim CM, Gomes KM, Reus GZ, Petronilho F, Ferreira GK, Streck EL, Dal-Pizzol F, Quevedo J (2014) Methylphenidate treatment causes oxidative stress and alters energetic metabolism in an animal model of attention-deficit hyperactivity disorder. Acta Neuropsychiatr 26:96-103.

Cortese S (2012) The neurobiology and genetics of Attention-Deficit/Hyperactivity Disorder (ADHD): what every clinician should know. Eur J Paediatr Neurol 16:422-433.

Cortese S, Kelly C, Chabernaud C, Proal E, Di Martino A, Milham MP, Castellanos FX (2012) Toward systems neuroscience of ADHD: a meta-analysis of 55 fMRI studies. Am J Psychiatry 169:1038-1055.

Costa VM, Carvalho F, Bastos ML, Carvalho RA, Carvalho M, Remiao F (2011) Contribution of catecholamine reactive intermediates and oxidative stress to the pathologic features of heart diseases. Curr Med Chem 18:2272-2314.

Costa VM, Carvalho F, Duarte JA, Bastos ML, Remiao F (2013) The heart as a target for xenobiotic toxicity: the cardiac susceptibility to oxidative stress. Chem Res Toxicol 26:1285-1311.

Costa VM, Silva R, Ferreira LM, Branco PS, Carvalho F, Bastos ML, Carvalho RA, Carvalho M, Remiao F (2007) Oxidation process of adrenaline in freshly isolated rat cardiomyocytes: formation of adrenochrome, quinoproteins, and GSH adduct. Chem Res Toxicol 20:1183-1191.

Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A (2006) Protein carbonylation, cellular dysfunction, and disease progression. J Cell Mol Med 10:389-406.

Daly MW, Custer G, McLeay PD (2008) Cardiac arrest with pulseless electrical activity associated with methylphenidate in an adolescent with a normal baseline echocardiogram. Pharmacotherapy 28:1408-1412.

DEA (2016) Drugs Schedules. Retrieved 26-08-2016, from https://www.dea.gov/druginfo/ds.shtml.

Ding YS, Gatley SJ, Thanos PK, Shea C, Garza V, Xu Y, Carter P, King P, Warner D, Taintor NB, Park DJ, Pyatt B, Fowler JS, Volkow ND (2004) Brain kinetics of methylphenidate (Ritalin) enantiomers after oral administration. Synapse 53:168-175.

Dopheide JA, Pliszka SR (2009) Attention-deficit-hyperactivity disorder: an update. Pharmacotherapy 29:656-679.

Egger H, Bartlett F, Dreyfuss R, Karliner J (1981) Metabolism of methylphenidate in dog and rat. Drug Metab Dispos 9:415-423.

EMEA (2009) Questions and answers on the review of medicines containing methylphenidate. Retrieved 26-08-2016, from http://www.ema.europa.eu/docs/en_GB/document_library/Referrals_document/Methylphenidate_31/WC500011125.pdf.

Fagundes AO, Aguiar MR, Aguiar CS, Scaini G, Sachet MU, Bernhardt NM, Rezin GT, Valvassori SS, Quevedo J, Streck EL (2010a) Effect of acute and chronic administration of methylphenidate on mitochondrial respiratory chain in the brain of young rats. Neurochem Res 35:1675-1680.

Fagundes AO, Scaini G, Santos PM, Sachet MU, Bernhardt NM, Rezin GT, Valvassori SS, Schuck PF, Quevedo J, Streck EL (2010b) Inhibition of mitochondrial respiratory chain in the brain of adult rats after acute and chronic administration of methylphenidate. Neurochem Res 35:405-411.

Faraj BA, Israili ZH, Perel JM, Jenkins ML, Holtzman SG, Cucinell SA, Dayton PG (1974) Metabolism and disposition of methylphenidate-14C: studies in man and animals. J Pharmacol Exp Ther 191:535-547.

Faraone SV, Bonvicini C, Scassellati C (2014) Biomarkers in the diagnosis of ADHD-promising directions. Curr Psychiatry Rep 16:497.

Faraone SV, Perlis RH, Doyle AE, Smoller JW, Goralnick JJ, Holmgren MA, Sklar P (2005) Molecular genetics of attention-deficit/hyperactivity disorder. Biol Psychiatry 57:1313-1323.

FDA (2006) Follow up review of AERS search identifying cases of sudden death occurring with drugs used for the treatment of Attention Deficit Hyperactivity Disorder (ADHD). Retrieved 28-08-2016, from http://www.fda.gov/ohrms/dockets/ac/06/briefing/2006-4210b_07_01_safetyreview.pdf.

FDA (2009a) CONCERTA ® (methylphenidate HCl) Extended-release Tablets CII. Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2007/021121s014lbl.pdf.

FDA (2009b) Metadate CD ® (methylphenidate HCl) Extended-Release Capsules. Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/021259s023lbl.pdf.

FDA (2009c) Methylin ® Chewable Tablets. Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2003/21475_methylin_lbl.pdf.

FDA (2010) Pediatric Studies Characteristics - Detail. Retrieved 10-08-2016, from http://www.accessdata.fda.gov/scripts/SDA/sdDetailNavigation.cfm?sd=fdaaadescriptorssortablewebdatabase&id=BE209EA4DA448BAEE040A8C0744D5262&rownum=2.

FDA (2011) Focalin XR (dexmethylphenidate hydrochloride) Extended-Release. Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/021802s022lbl.pdf.

FDA (2013a) Focalin ® (dexmethylphenidate hydrochloride tablets).Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2013/021278s016lbl.pdf.

FDA (2013b) Ritalin ®, Ritalin SR ® and Ritalin LA ® (methylphenidate hydrochloride).Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/010187s080,018029s049,021284s027lbl.pdf.

FDA (2015) Daytrana ® (methylphenidate transdermal system). Retrieved 14-08-2016, from http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/021514s023lbl.pdf.

Fettahoglu EC, Satilmis A, Gokcen C, Ozatalay E (2009) Oral megadose methylphenidate ingestion for suicide attempt. Pediatr Int 51:844-845.

Foley R, Mrvos R, Krenzelok EP (2000) A profile of methylphenidate exposures. J Toxicol Clin Toxicol 38:625-630.

Geissler J, Lesch KP (2011) A lifetime of attention-deficit/hyperactivity disorder: diagnostic challenges, treatment and neurobiological mechanisms. Expert Rev Neurother 11:1467-1484.

Gizer IR, Ficks C, Waldman ID (2009) Candidate gene studies of ADHD: a meta-analytic review. Hum Genet 126:51-90.

Gomes KM, Inacio CG, Valvassori SS, Reus GZ, Boeck CR, Dal-Pizzol F, Quevedo J (2009) Superoxide production after acute and chronic treatment with methylphenidate in young and adult rats. Neurosci Lett 465:95-98.

Gomes KM, Petronilho FC, Mantovani M, Garbelotto T, Boeck CR, Dal-Pizzol F, Quevedo J (2008) Antioxidant enzyme activities following acute or chronic methylphenidate treatment in young rats. Neurochem Res 33:1024-1027.

Gormley L, Turner A, Freeland K (2014) Clonidine and guanfacine IR vs ER: Old drugs with “new” formulations. Mental Health Clinician 4:22-26.

Gray JD, Punsoni M, Tabori NE, Melton JT, Fanslow V, Ward MJ, Zupan B, Menzer D, Rice J, Drake CT, Romeo RD, Brake WG, Torres-Reveron A, Milner TA (2007) Methylphenidate administration to juvenile rats alters brain areas involved in cognition, motivated behaviors, appetite, and stress. J Neurosci 27:7196-7207.

Heal DJ, Pierce DM (2006) Methylphenidate and its isomers: their role in the treatment of attention-deficit hyperactivity disorder using a transdermal delivery system. CNS Drugs 20:713-738.

Henderson TA, Fischer VW (1995) Effects of methylphenidate (Ritalin) on mammalian myocardial ultrastructure. Am J Cardiovasc Pathol 5:68-78.

Hungund BL, Perel JM, Hurwic MJ, Sverd J, Winsberg BG (1979) Pharmacokinetics of methylphenidate in hyperkinetic children. Br J Clin Pharmacol 8:571-576.

Kaplan G, Newcorn JH (2011) Pharmacotherapy for child and adolescent attention-deficit hyperactivity disorder. Pediatr Clin North Am 58:99-120, xi.

Kimko HC, Cross JT, Abernethy DR (1999) Pharmacokinetics and clinical effectiveness of methylphenidate. Clin Pharmacokinet 37:457-470.

Klampfl K, Quattlander A, Burger R, Pfuhlmann B, Warnke A, Gerlach M (2010) Case report: intoxication with high dose of long-acting methylphenidate (Concerta (R®)) in a suicidal 14-year-old girl. Atten Defic Hyperact Disord 2:221-224.

Kuczenski R, Segal DS (2002) Exposure of adolescent rats to oral methylphenidate: preferential effects on extracellular norepinephrine and absence of sensitization and cross-sensitization to methamphetamine. J Neurosci 22:7264-7271.

Lange KW, Reichl S, Lange KM, Tucha L, Tucha O (2010) The history of attention deficit hyperactivity disorder. Atten Defic Hyperact Disord 2:241-255.

Laughren TP (2009) Memorandum. Retrieved 10-09-2016, from http://www.fda.gov/downloads/drugs/developmentapprovalprocess/developmentresources/ucm226659.pdf.

Leonard BE, McCartan D, White J, King DJ (2004) Methylphenidate: a review of its neuropharmacological, neuropsychological and adverse clinical effects. Hum Psychopharmacol 19:151-180.

Leuchter AF, McGough JJ, Korb AS, Hunter AM, Glaser PE, Deldar A, Durell TM, Cook IA (2014) Neurophysiologic predictors of response to atomoxetine in young adults with attention deficit hyperactivity disorder: a pilot project. J Psychiatr Res 54:11-18.

Levi MS, Divine B, Hanig JP, Doerge DR, Vanlandingham MM, George NI, Twaddle NC, Bowyer JF (2012) A comparison of methylphenidate-, amphetamine-, and methamphetamine-induced hyperthermia and neurotoxicity in male Sprague-Dawley rats during the waking (lights off) cycle. Neurotoxicol Teratol 34:253-262.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265-275.

Lucas PB, Gardner DL, Wolkowitz OM, Tucker EE, Cowdry RW (1986) Methylphenidate-induced cardiac arrhythmias. N Engl J Med 315:1485.

Madras BK, Miller GM, Fischman AJ (2005) The dopamine transporter and attention-deficit/hyperactivity disorder. Biol Psychiatry 57:1397-1409.

Maneeton N, Maneeton B, Intaprasert S, Woottiluk P (2014) A systematic review of randomized controlled trials of bupropion versus methylphenidate in the treatment of attention-deficit/hyperactivity disorder. Neuropsychiatr Dis Treat 10:1439-1449.

Marco EM, Adriani W, Ruocco LA, Canese R, Sadile AG, Laviola G (2011) Neurobehavioral adaptations to methylphenidate: the issue of early adolescent exposure. Neurosci Biobehav Rev 35:1722-1739.

Martins MR, Reinke A, Petronilho FC, Gomes KM, Dal-Pizzol F, Quevedo J (2006) Methylphenidate treatment induces oxidative stress in young rat brain. Brain Res 1078:189-197.

Matthews M, Nigg JT, Fair DA (2014) Attention deficit hyperactivity disorder. Curr Top Behav Neurosci 16:235-266.

Mehta H, Murray B, Loludice TA (1984) Hepatic dysfunction due to intravenous abuse of methylphenidate hydrochloride. J Clin Gastroenterol 6:149-151.

Mick E, Faraone SV (2008) Genetics of attention deficit hyperactivity disorder. Child Adolesc Psychiatr Clin N Am 17:261-284, vii-viii.

Mick E, McManus DD, Goldberg RJ (2013) Meta-analysis of increased heart rate and blood pressure associated with CNS stimulant treatment of ADHD in adults. Eur Neuropsychopharmacol 23:534-541.

Moll GH, Hause S, Ruther E, Rothenberger A, Huether G (2001) Early methylphenidate administration to young rats causes a persistent reduction in the density of striatal dopamine transporters. J Child Adolesc Psychopharmacol 11:15-24.

Morton WA, Stockton GG (2000) Methylphenidate abuse and psychiatric side effects. Prim Care Companion J Clin Psychiatry 2:159-164.

Motaghinejad M, Motevalian M, Shabab B (2016) Effects of chronic treatment with methylphenidate on oxidative stress and inflammation in hippocampus of adult rats. Neurosci Lett 619:106-113.

Munk K, Gormsen L, Kim WY, Andersen NH (2015) Cardiac arrest following a myocardial infarction in a child treated with methylphenidate. Case Rep Pediatr 2015:905097.

Murat D, Arman A (2013) Suicidal attempt with high dose long-acting methylphenidate: a case report. Marmara Medical Journal 26:165-167.

Nakao T, Radua J, Rubia K, Mataix-Cols D (2011) Gray matter volume abnormalities in ADHD: voxel-based meta-analysis exploring the effects of age and stimulant medication. Am J Psychiatry 168:1154-1163.

Nigg J, Nikolas M, Burt SA (2010) Measured gene-by-environment interaction in relation to attention-deficit/hyperactivity disorder. J Am Acad Child Adolesc Psychiatry 49:863-873.

Nymark TB, Hovland A, Bjornstad H, Nielsen EW (2008) A young man with acute dilated cardiomyopathy associated with methylphenidate. Vasc Health Risk Manag 4:477-479.

Owen JB, Butterfield DA (2010) Measurement of oxidized/reduced glutathione ratio. Methods Mol Biol 648:269-277.

Ozdemir E, Karaman MG, Yurteri N, Erdogan A (2010) A case of suicide attempt with long-acting methylphenidate (Concerta). Atten Defic Hyperact Disord 2:103-105.

Patrick KS, Ellington KR, Breese GR (1984) Distribution of methylphenidate and p-hydroxymethylphenidate in rats. J Pharmacol Exp Ther 231:61-65.

Peyre H, Delorme R (2012) A case of severe hyperthermia after administration of methylphenidate. J Clin Psychopharmacol 32:299-300.

Polanczyk G, de Lima MS, Horta BL, Biederman J, Rohde LA (2007) The worldwide prevalence of ADHD: a systematic review and metaregression analysis. Am J Psychiatry 164:942-948.

Popper CW (1997) Antidepressants in the treatment of attention-deficit/hyperactivity disorder. J Clin Psychiatry 58 Suppl 14:14-29; discussion 30-11.

Porfirio MC, Giana G, Giovinazzo S, Curatolo P (2011) Methylphenidate-induced visual hallucinations. Neuropediatrics 42:30-31.

Prince J (2008) Catecholamine dysfunction in attention-deficit/hyperactivity disorder: an update. J Clin Psychopharmacol 28:S39-45.

Pucak ML, Grace AA (1994) Regulation of substantia nigra dopamine neurons. Crit Rev Neurobiol 9:67-89.

Ramaekers JG, Evers EA, Theunissen EL, Kuypers KP, Goulas A, Stiers P (2013) Methylphenidate reduces functional connectivity of nucleus accumbens in brain reward circuit. Psychopharmacology (Berl) 229:219-226.

Rapport MD, Moffitt C (2002) Attention deficit/hyperactivity disorder and methylphenidate. A review of height/weight, cardiovascular, and somatic complaint side effects. Clin Psychol Rev 22:1107-1131.

Redalieu E, Bartlett MF, Waldes LM, Darrow WR, Egger H, Wagner WE (1982) A study of methylphenidate in man with respect to its major metabolite. Drug Metab Dispos 10:708-709.

Reus GZ, Scaini G, Furlanetto CB, Morais MO, Jeremias IC, Mello-Santos LM, Freitas KV, Quevedo J, Streck EL (2013) Methylphenidate treatment leads to abnormalities on krebs cycle enzymes in the brain of young and adult rats. Neurotox Res 24:251-257.

Roberts SM, Harbison RD, Roth L, James RC (1994) Methylphenidate-induced hepatotoxicity in mice and its potentiation by beta-adrenergic agonist drugs. Life Sci 55:269-281.

Rubia K, Overmeyer S, Taylor E, Brammer M, Williams SC, Simmons A, Bullmore ET (1999) Hypofrontality in attention deficit hyperactivity disorder during higher-order motor control: a study with functional MRI. Am J Psychiatry 156:891-896.

Salviano LH, Linhares MI, de Lima KA, de Souza AG, Lima DB, Jorge AR, da Costa MF, Filho AJ, Martins AM, Monteiro HS, de Jesus Ponte Carvalho TM, de Franca

Fonteles MM (2015) Study of the safety of methylphenidate: Focus on nephrotoxicity aspects. Life Sci 141:137-142.

Scharman EJ, Erdman AR, Cobaugh DJ, Olson KR, Woolf AD, Caravati EM, Chyka PA, Booze LL, Manoguerra AS, Nelson LS, Christianson G, Troutman WG, American Association of Poison Control C (2007) Methylphenidate poisoning: an evidence-based consensus guideline for out-of-hospital management. Clin Toxicol (Phila) 45:737-752.

Scherer EB, da Cunha MJ, Matte C, Schmitz F, Netto CA, Wyse AT (2010) Methylphenidate affects memory, brain-derived neurotrophic factor immunocontent and brain acetylcholinesterase activity in the rat. Neurobiol Learn Mem 94:247-253.

Schmitz F, Pierozan P, Rodrigues AF, Biasibetti H, Grunevald M, Pettenuzzo LF, Scaini G, Streck EL, Netto CA, Wyse AT (2016) Methylphenidate Causes Behavioral Impairments and Neuron and Astrocyte Loss in the Hippocampus of Juvenile Rats. Mol Neurobiol.

Schmitz F, Scherer EB, Machado FR, da Cunha AA, Tagliari B, Netto CA, Wyse AT (2012) Methylphenidate induces lipid and protein damage in prefrontal cortex, but not in cerebellum, striatum and hippocampus of juvenile rats. Metab Brain Dis 27:605-612.

Schteinschnaider A, Plaghos LL, Garbugino S, Riveros D, Lazarowski A, Intruvini S, Massaro M (2000) Cerebral arteritis following methylphenidate use. J Child Neurol 15:265-267.

Schweren LJ, de Zeeuw P, Durston S (2013) MR imaging of the effects of methylphenidate on brain structure and function in attention-deficit/hyperactivity disorder. Eur Neuropsychopharmacol 23:1151-1164.

Sharma A, Couture J (2014) A review of the pathophysiology, etiology, and treatment of attention-deficit hyperactivity disorder (ADHD). Ann Pharmacother 48:209-225.

Shaw P, Eckstrand K, Sharp W, Blumenthal J, Lerch JP, Greenstein D, Clasen L, Evans A, Giedd J, Rapoport JL (2007) Attention-deficit/hyperactivity disorder is characterized by a delay in cortical maturation. Proc Natl Acad Sci U S A 104:19649-19654.

Shaw P, Lerch J, Greenstein D, Sharp W, Clasen L, Evans A, Giedd J, Castellanos FX, Rapoport J (2006) Longitudinal mapping of cortical thickness and clinical outcome in children and adolescents with attention-deficit/hyperactivity disorder. Arch Gen Psychiatry 63:540-549.

Sheridan MA, Hinshaw S, D'Esposito M (2010) Stimulant medication and prefrontal functional connectivity during working memory in ADHD: a preliminary report. J Atten Disord 14:69-78.

Shram MJ, Quinn AM, Chen N, Faulknor J, Luong D, Sellers EM, Endrenyi L (2012) Differences in the in vitro and in vivo pharmacokinetic profiles of once-daily modified-release methylphenidate formulations in Canada: examination of current bioequivalence criteria. Clin Ther 34:1170-1181.

Simon V, Czobor P, Balint S, Meszaros A, Bitter I (2009) Prevalence and correlates of adult attention-deficit hyperactivity disorder: meta-analysis. Br J Psychiatry 194:204-211.

Spear LP (2000) The adolescent brain and age-related behavioral manifestations. Neurosci Biobehav Rev 24:417-463.

Spencer T, Biederman J, Wilens T, Harding M, O'Donnell D, Griffin S (1996) Pharmacotherapy of attention-deficit hyperactivity disorder across the life cycle. J Am Acad Child Adolesc Psychiatry 35:409-432.

Spencer TJ, Brown A, Seidman LJ, Valera EM, Makris N, Lomedico A, Faraone SV, Biederman J (2013) Effect of psychostimulants on brain structure and function in ADHD: a qualitative literature review of magnetic resonance imaging-based neuroimaging studies. J Clin Psychiatry 74:902-917.

Srinivas NR, Hubbard JW, Korchinski ED, Midha KK (1992) Stereoselective urinary pharmacokinetics of dl-threo-methylphenidate and its major metabolite in humans. J Pharm Sci 81:747-749.

Srinivas NR, Hubbard JW, Korchinski ED, Midha KK (1993) Enantioselective pharmacokinetics of dl-threo-methylphenidate in humans. Pharm Res 10:14-21.

Stecyk O, Loludice TA, Demeter S, Jacobs J (1985) Multiple organ failure resulting from intravenous abuse of methylphenidate hydrochloride. Ann Emerg Med 14:597-599.

Stiefel G, Besag FM (2010) Cardiovascular effects of methylphenidate, amphetamines and atomoxetine in the treatment of attention-deficit hyperactivity disorder. Drug Saf 33:821-842.

Still GF (1902) Some abnormal psychical conditions in children: the Goulstonian Lectures. Lancet 1:1008 - 1012.

Sugrue D, Bogner R, Ehret MJ (2014) Methylphenidate and dexmethylphenidate formulations for children with attention-deficit/hyperactivity disorder. Am J Health Syst Pharm 71:1163-1170.

Sun Z, Murry DJ, Sanghani SP, Davis WI, Kedishvili NY, Zou Q, Hurley TD, Bosron WF (2004) Methylphenidate is stereoselectively hydrolyzed by human carboxylesterase CES1A1. J Pharmacol Exp Ther 310:469-476.

Swanson JM, Volkow ND (2002) Pharmacokinetic and pharmacodynamic properties of stimulants: implications for the design of new treatments for ADHD. Behav Brain Res 130:73-78.

Swanson LW (2004) Brain maps: structure of the rat brain: Oxford: Elsevier Academic Press.

Take G, Bahcelioglu M, Oktem H, Tunc E, Gozil R, Erdogan D, Calguner E, Helvacioglu F, Giray SG, Elmas C (2008) Dose-dependent immunohistochemical and ultrastructural changes after oral methylphenidate administration in rat heart tissue. Anat Histol Embryol 37:303-308.

Tarver J, Daley D, Sayal K (2014) Attention-deficit hyperactivity disorder (ADHD): an updated review of the essential facts. Child Care Health Dev 40:762-774.

Teixeira-Gomes A, Costa VM, Feio-Azevedo R, Bastos Mde L, Carvalho F, Capela JP (2015) The neurotoxicity of amphetamines during the adolescent period. Int J Dev Neurosci 41:44-62.

Teixeira-Gomes A, Costa VM, Feio-Azevedo R, Duarte JA, Duarte-Araujo M, Fernandes E, Bastos ML, Carvalho F, Capela JP (2016) "Ecstasy" toxicity to adolescent rats following an acute low binge dose. BMC Pharmacol Toxicol 17:28.

Thanos PK, Robison LS, Steier J, Hwang YF, Cooper T, Swanson JM, Komatsu DE, Hadjiargyrou M, Volkow ND (2015) A pharmacokinetic model of oral methylphenidate in the rat and effects on behavior. Pharmacol Biochem Behav 131:143-153.

Thompson J, Thompson JR (2010) Acute myocardial infarction related to methylphenidate for adult attention deficit disorder. J Emerg Med 38:18-21.

Trugman JM (1988) Cerebral arteritis and oral methylphenidate. Lancet 1:584-585.

Ueha S, Shand FH, Matsushima K (2012) Cellular and molecular mechanisms of chronic inflammation-associated organ fibrosis. Front Immunol 3:71.

Valera EM, Faraone SV, Murray KE, Seidman LJ (2007) Meta-analysis of structural imaging findings in attention-deficit/hyperactivity disorder. Biol Psychiatry 61:1361-1369.

van der Marel K, Klomp A, Meerhoff GF, Schipper P, Lucassen PJ, Homberg JR, Dijkhuizen RM, Reneman L (2014) Long-term oral methylphenidate treatment in adolescent and adult rats: differential effects on brain morphology and function. Neuropsychopharmacology 39:263-273.

Volkow ND, Ding YS, Fowler JS, Wang GJ, Logan J, Gatley JS, Dewey S, Ashby C, Liebermann J, Hitzemann R, et al. (1995) Is methylphenidate like cocaine? Studies on their pharmacokinetics and distribution in the human brain. Arch Gen Psychiatry 52:456-463.

Volkow ND, Fowler JS, Wang GJ, Ding YS, Gatley SJ (2002) Role of dopamine in the therapeutic and reinforcing effects of methylphenidate in humans: results from imaging studies. Eur Neuropsychopharmacol 12:557-566.

Volkow ND, Wang GJ, Fowler JS, Ding YS (2005) Imaging the effects of methylphenidate on brain dopamine: new model on its therapeutic actions for attention-deficit/hyperactivity disorder. Biol Psychiatry 57:1410-1415.

Volkow ND, Wang GJ, Fowler JS, Molina PE, Logan J, Gatley SJ, Gifford A, Ding YS, Wong C, Pappas NR, Zhu W, Swanson JM (2003) Cardiovascular effects of methylphenidate in humans are associated with increases of dopamine in brain and of epinephrine in plasma. Psychopharmacology (Berl) 166:264-270.

Volz TJ (2008) Neuropharmacological mechanisms underlying the neuroprotective effects of methylphenidate. Curr Neuropharmacol 6:379-385.

Wang GJ, Volkow ND, Wigal T, Kollins SH, Newcorn JH, Telang F, Logan J, Jayne M, Wong CT, Han H, Fowler JS, Zhu W, Swanson JM (2013) Long-term stimulant treatment affects brain dopamine transporter level in patients with attention deficit hyperactive disorder. PLoS One 8:e63023.

Wargin W, Patrick K, Kilts C, Gualtieri CT, Ellington K, Mueller RA, Kraemer G, Breese GR (1983) Pharmacokinetics of methylphenidate in man, rat and monkey. J Pharmacol Exp Ther 226:382-386.

Weyandt L, Swentosky A, Gudmundsdottir BG (2013) Neuroimaging and ADHD: fMRI, PET, DTI findings, and methodological limitations. Dev Neuropsychol 38:211-225.

Wolraich ML, Doffing MA (2004) Pharmacokinetic considerations in the treatment of attention-deficit hyperactivity disorder with methylphenidate. CNS Drugs 18:243-250.

Wynn TA (2008) Cellular and molecular mechanisms of fibrosis. J Pathol 214:199-210.

Yang PB, Amini B, Swann AC, Dafny N (2003) Strain differences in the behavioral responses of male rats to chronically administered methylphenidate. Brain Res 971:139-152.

Zheng G, Dwoskin LP, Crooks PA (2006) Vesicular monoamine transporter 2: role as a novel target for drug development. The AAPS journal 8:E682-692.